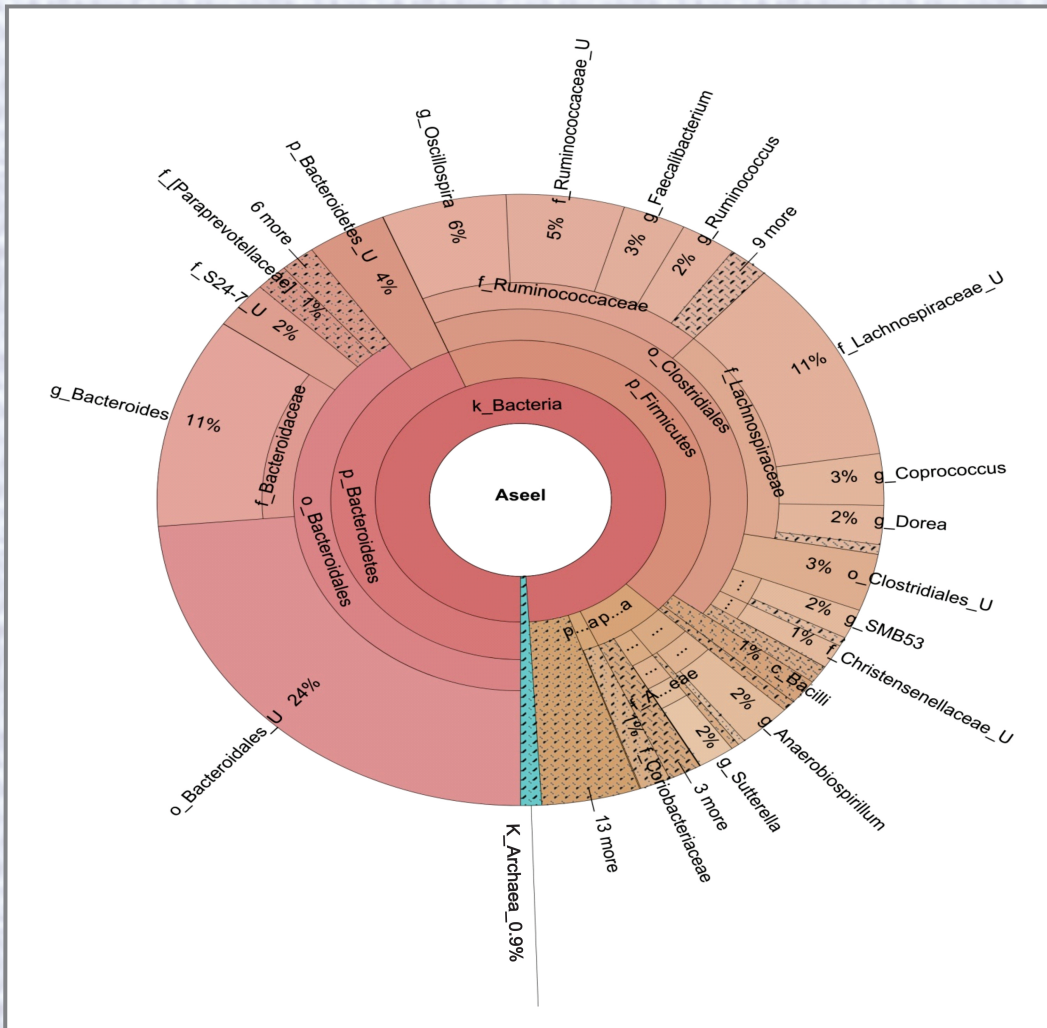


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Cover Page Photo : Krona chart of taxonomic assignments of 16S amplicon sequencing reads of metagenomic DNA of gut content of aseel breed of chicken indicating mean relative abundance of the prokaryotes at different taxonomic levels.

Avian pathogenic *Escherichia coli* infection in chickens: A brief review on pathogenesis, epidemiology, virulence, antibiotic resistance, pathology, diagnosis and control strategies

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ABSTRACT

Avian Pathogenic *Escherichia coli* (APEC) is a major hazard to the chicken industry globally, causing huge economic losses and threatening food security. This review covers major features of APEC infection in chickens, including pathogenesis, epidemiology, virulence genes, antibiotic resistance, pathology, diagnostics, and control measures. APEC pathogenesis is complicated by interactions between bacterial factors and host defences. It is critical to understand these mechanisms to design efficient control methods. Epidemiological findings investigate the prevalence, transmission patterns, and risk factors linked with APEC, offering light on the disease's global impact. Virulence genes have an important role in the pathogenicity of APEC, and their identification is critical for developing focused intervention techniques. The advent of antibiotic resistance in APEC hampers treatment choices even more, emphasizing the importance of antimicrobial prudence and alternate management techniques. Pathological signs of APEC infection are investigated, ranging from respiratory to systemic indications, to provide a foundation for appropriate diagnosis. The applicability and sensitivity of diagnostic procedures, such as molecular techniques and serological testing, are discussed. Finally, the review discusses current and emerging control techniques, including immunization, biosecurity, and probiotic therapies.

Keywords: Antibiotic Resistance, APEC, Control Strategies, Diagnosis, Epidemiology, Gross Pathology, Histopathology, One Health, Pathogenesis, Vaccine, Virulence

INTRODUCTION

Avian Pathogenic *Escherichia coli* (APEC) is a subset of the bacterium *Escherichia coli* that has been specifically adapted to infect avian species, most notably chickens. APEC continues to pose a severe danger to the global poultry sector, causing enormous economic losses and threatening food security. Extensive research has been undertaken over the past decades to unravel the complexities of APEC infection, covering topics like as pathogenesis, epidemiology, virulence genes, antibiotic resistance, pathology, diagnosis, and control measures (Krishnegowda *et al.*, 2020; Kathayat *et al.*, 2021; Krishnegowda *et al.*, 2022; Ovi *et al.*, 2023). Understanding APEC is becoming increasingly important for the development of effective preventive and treatment approaches as the chicken industry evolves and intensifies rampantly.

The pathophysiology of APEC infection involves a complicated interplay of bacterial components and host responses, which influences the disease's severity and prognosis. Recent reports have revealed novel strategies used by APEC to elude host defences, highlighting the need for further investigation of these complicated pathways (Kathayat *et al.*, 2021). Epidemiological knowledge of APEC prevalence, transmission dynamics, and risk factors is required for the implementation of tailored control measures. The global outbreaks and the introduction of new APEC strains highlight the pathogen's dynamic nature, demanding continuing surveillance (Ewers *et al.*, 2004; chen *et al.*, 2021). Virulence genes are important in APEC pathogenicity, and recent advances in

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molecular biology have allowed for the identification and characterization of major genetic factors. The investigation of these virulence factors gives essential insights into APEC pathogenesis as well as prospective therapeutic targets (Mellata *et al.*, 2010; Hu *et al.*, 2022). The growing concern about antibiotic resistance in APEC strains complicates infection management and necessitates the prudent use of antimicrobials. Recent research

into the genetic basis of antibiotic resistance in APEC has highlighted the critical need for alternate therapeutic techniques (Kabiswa *et al.*, 2018; Afayibo *et al.*, 2022; Bastidas-Caldes *et al.*, 2022). Pathological signs of APEC infection, ranging from localized respiratory infections to systemic infections, add to the disease's complexity. The pathological studies have shed light on tissue-specific responses as well as the dynamics of APEC dissemination throughout avian hosts, enabling diagnostic techniques and therapeutic interventions (Krishnegowda *et al.*, 2020; Mehat *et al.*, 2021; Krishnegowda *et al.*, 2022). Diagnosis of APEC infections must be accurate and prompt to apply effective control strategies. Recent advancements in diagnostic technology, such as polymerase chain reaction (PCR) assays and serological approaches, provide increased sensitivity and specificity in diagnosing APEC, allowing for faster response and containment tactics (Schouler *et al.*, 2012; Wilczyński *et al.*, 2022).

In light of these difficulties, the purpose of this review is to summarise recent advances in our understanding of APEC infection in chickens. This review provides a comprehensive overview of pathogenesis, epidemiology, virulence genes, antibiotic resistance, pathology, diagnosis, and control strategies by referring to the recent literature. Also provides a complete perspective, which is required for the development of integrated and long-term measures to mitigate the impact of APEC on chicken health and production.

EPIDEMIOLOGY

APEC epidemiology is a complex interplay of several factors that determine the emergence, transmission, and persistence of these pathogenic strains within avian populations. The host vulnerability, environmental variables, and the probable significance of horizontal gene transfer in the evolution of virulence features are all discussed here.

Host Susceptibility

The susceptibility of avian hosts is an important feature of APEC epidemiology. The likelihood of infection is influenced by factors such as age, immunological condition, and overall health. Though all the age groups are affected, young chicks and stressed birds are especially vulnerable to the infection (Kathayat *et al.*, 2021).

Environmental factors

The environment is critical to APEC transmission. Dziva and Stevens (2008) attribute the survival of APEC in chicken farms to contaminated water supplies, overcrowding, and poor sanitation. Christensen *et al.*, (2021) highlight the necessity for stronger biosecurity efforts to address environmental risk factors.

Genetic Diversity and Evolution

APEC strain genetic variability provides another element of its epidemiology. Recent genomic investigations have identified a variety of virulence factors and mobile genetic components that contribute to APEC's adaptability and evolution (Mageiros *et al.*, 2021). This genetic diversity highlights the importance of continued surveillance to monitor strain variations.

Horizontal Gene Transfer

The movement of genetic material between bacteria via horizontal gene transfer is a major feature in APEC epidemiology. Mobile genetic factors like plasmids and bacteriophages have a role in the spread of virulence and antibiotic-resistance genes among APEC strains (Johnson *et al.*, 2007; Pilati *et al.*, 2023). Understanding these pathways is critical for forecasting and responding to APEC outbreaks.

Recent studies have considerably improved our understanding of APEC epidemiology. Chen *et al.*, (2021), for example, explored the molecular epidemiology of APEC in Chinese chicken farms, emphasizing the need to monitor genetic alterations and the effect of horizontal gene transmission.

In conclusion, APEC epidemiology is a complicated problem driven by host characteristics, environmental conditions, and the changing nature of bacterial genetics. Research demonstrates the importance of ongoing research and surveillance efforts in creating effective control and prevention techniques to protect poultry health.

PATHOGENESIS

The molecular processes that underpin APEC pathogenesis are briefed in this section. It discusses key virulence elements like adhesins, toxins, and iron acquisition systems that allow APEC to colonize and infect the avian host and also the connection between APEC and the chicken immune system. The potential of APEC to cause colibacillosis, a complex disease in chicken, distinguishes it. The pathogenesis of APEC is complicated by the interaction of bacterial virulence factors, host responses, and environmental variables. The research has made tremendous progress in understanding the molecular pathways underlying APEC pathogenesis (Dziva and Stevens 2008). To produce colibacillosis in chicken, APEC either possesses or uses distinct virulence and pathogenesis components or pathways. Adhesins, invasins, protectins, iron acquisition systems, toxins, two-component systems, quorum-sensing (QS) systems, transcriptional regulators, secretion systems, and metabolism-related genes are a few examples of these components (Kathayat *et al.*, 2021).

Adhesion and Colonization

In chickens, APEC causes infection by adhering to

and colonizing the mucosal surfaces of the respiratory and gastrointestinal systems as well as other visceral organs. Adhesins, such as Type 1 and P fimbriae, play an important role in mediating bacterial adhesion to host cells (Dziva and Stevens 2008). Studies have highlighted the variability of adhesin profiles among APEC strains, which contributes to variances in host tissue tropism (Ali *et al.*, 2020; Aleksandrowicz *et al.*, 2021).

Invasion and Survival

APEC strains have a virulence arsenal that allows them to infiltrate host tissues. The ability of APEC to persist and replicate among macrophages and other immune cells is critical to its pathogenesis (Mellata *et al.*, 2003). Outer membrane proteins (*ompT*), including TraT and IbeA, have been reported as key contributors to APEC intracellular survival (Ewers *et al.*, 2003; Ewers *et al.*, 2007; Hejair *et al.*, 2017; Kathayat *et al.*, 2021).

Toxin Production

Toxins produced by extraintestinal *E. coli* contribute to tissue damage and disease progression. Garcia *et al.*, (2013) cite cytotoxic necrotizing factor 1 (CNF1) and hemolysin as examples of proteins implicated in host cell damage. Kathayat *et al.*, (2021) reported the regulation and expression of toxin genes and different toxins and their role in APEC pathogenesis. EAST-1 (heat-stable enterotoxin), *astA*, *pic* (serine protease autotransporter), *espC* (serine protease), *ace4/35* (acetylcholine esterase), *hlyF*, *hlyA*, *hlyE* (putative avian hemolysin), *vat* (vacuolating autotransporter toxin), *sat* (secreted autotransporter toxin), *cdtB*, *cdtS* (cytolethal distending factor), and *astA* have all been reported in APEC (Kathayat *et al.*, 2021). In addition, these toxins promote the development of outer membrane vesicles, colonization, motility, biofilm formation, agglutination, and induction of vacuolization (Murase *et al.*, 2016).

Iron Acquisition

When it comes to obtaining vital nutrients from the host—iron in particular—APEC is incredibly versatile. Iron acquisition systems like *sitABCD* and *iutA*, along with siderophores, enable APEC to compete for the scarce iron resources in the host environment. The regulations of these systems are important in APEC pathogenicity (Gao *et al.*, 2012; Schouler *et al.*, 2012).

Host Immune Response:

APEC's interaction with the host immune system is dynamic and complicated. APEC can modify the host's inflammatory response, elude the immune system, and survive in the host (Azeem *et al.*, 2017). Cirl *et al.*, (2008) focused on identifying the host variables that determine susceptibility to extraintestinal *E. coli* infections, providing information on the complex balance between bacterial pathogenicity and host defense mechanisms.

To summarise, APEC pathogenesis encompasses

a complex set of molecular processes that allow the bacteria to establish infection, avoid host defenses, and cause disease in chickens. Continuous research efforts are required for a full understanding of these pathways, which will eventually lead to the development of targeted therapies against APEC infections.

VIRULENCE FACTORS

APEC possesses a wide range of virulence characteristics that contribute to its potential to induce colibacillosis in poultry. Understanding the molecular mechanisms underlying these virulence factors is critical for knowing the pathophysiology of APEC. The following are the imperative virulence factors in APEC

Adhesins

Adhesins, which are essential for bacterial adhesion, enhance APEC's initial contact with host tissues. Dziva and Stevens (2008) list type 1 fimbriae, P fimbriae, and curli fibres as well-studied APEC adhesins. Recent genomic investigations have indicated the presence of various adhesin patterns among APEC strains, which may contribute to differences in tissue tropism and host specificity (Ali *et al.*, 2020). Every type of adhesin—including fimbrial chaperone-usher, fimbrial curli, nonfimbrial, and atypical adhesins - as well as their constituent parts are involved in pathogenesis. Although some adhesins, like S, F1C, Dr, and type IV fimbriae, have already been identified and found in APEC genomes, their functions in virulence have not yet been explored. FdeC and Ygi fimbriae, on the other hand, have been investigated very often. (Aleksandrowicz *et al.*, 2021).

Iron Uptake Systems

Iron acquisition from the host environment must be efficient for APEC survival. Aerobactin and other siderophores, as well as iron uptake systems like SitABCD and IutA, play critical roles in pathogenesis. The studies reported the regulatory mechanisms that govern these systems, revealing information on their importance in APEC pathogenicity. Once bacteria have effectively colonized and/or invaded the host, iron is a crucial micronutrient needed for the growth and multiplication of the bacteria inside the host cell. To sequester iron from bodily fluids, APEC has many iron acquisition systems that include transporters and several siderophores, including yersiniabactin, salmochelin, and aerobactin (Kathayat *et al.*, 2021). Aerobactin and salmochelin systems were found to be significant in APEC pathogenicity, with salmochelin having a greater effect on virulence. (Rodriguez-Siek *et al.*, 2005; Gao *et al.*, 2012; Niu *et al.*, 2022).

Toxins

As we have discussed previously the various toxins produced by APEC contribute to tissue damage and

disease progression. Garcia *et al.*, (2013) found that cytotoxic necrotizing factor 1 (CNF1) and hemolysin are also involved in host cell damage. Because of advances in molecular biology, we now have a better understanding of toxin regulation and expression, as well as insights into their function in APEC pathogenesis (Kathayat *et al.*, 2021).

Capsule Formation

The development of a capsule is an important virulence mechanism that allows APEC to avoid host immune responses. Polysaccharides in the capsular membrane protect bacteria against phagocytosis and complement-mediated death. Thereby capsule acts as a protectin that protects bacteria from various unfavourable conditions within the host (Mellata *et al.*, 2003; Kathayat *et al.*, 2021). The diversity of capsule types among APEC strains establishes a relationship between particular forms and pathogenicity (Ewers *et al.*, 2003; Ewers *et al.*, 2007).

Invasins

Invasins are a class of proteins crucial for facilitating the entry of bacteria into the host cell in the early stages of infection (Kathayat *et al.*, 2021). Many outer membrane proteins as briefed earlier involved in APEC survival and proliferate within macrophages (Hejair *et al.*, 2017).

Type VI Secretion System (T6SS)

A multiprotein complex called the type VI secretion system (T6SS) is responsible for delivering toxins to both bacterial and eukaryotic cells. As such, it takes part in both pathogenesis and interbacterial competition. Hence T6SS is a very imperative virulence factor that is involved in bacterial and host interactions. APEC strains have a functioning T6SS that may play a role in pathogenicity. T6SS's significance in APEC infections and interactions with other virulence factors have been studied (Journet and Cascales, 2016). It has been shown that the VgrG protein, an essential part and effector of T6SS, has a variety of functions. VgrG protein's N-terminal domain is a homolog of phage T4's tail fibre protein, gp27, which carries out receptor binding and establishes host specificity. (Ma *et al.*, 2014). As described above, many virulence factors are used by APEC to cause infection in chickens. Studies are necessary to have a thorough understanding of the regulatory networks that govern these factors, perhaps paving the path for specific therapies to control APEC infections in poultry.

VIRULENCE GENES

APEC possesses a complex set of virulence genes that code for specific virulence factors involved in pathogenesis and pathology that cause disease in chickens. (Kathayat *et al.*, 2021; Bhattarai *et al.*, 2023; Ovi *et al.*, 2023). This section provides an overview of the

virulence genes in APEC, emphasizing recent findings that deepen our understanding of the molecular basis of APEC pathogenesis. Continued research into these genes and their regulation is crucial for the development of targeted strategies to mitigate APEC-associated diseases in poultry.

The following are the few imperative virulence genes in APEC

Fimbrial Adhesin Genes

The ability of APEC to adhere to host tissues is critical to its pathogenicity. Fimbrial adhesins, such as Type 1 and P fimbriae, facilitate adherence to specific host cell receptors (Dziva and Stevens 2008). The genomic investigations have identified differences in fimbrial gene profiles among APEC strains, which influence host tissue tropism and pathogenicity. The APEC genes *fdtA*, *rluD*, *yjhB*, *ecpR*, and *fdeC* were found to have roles in adhesion to human and chicken intestinal cell lines. The prevention and management of APEC infections will benefit from the identification of these genes' roles in APEC pathogenesis (Ali *et al.*, 2020; Aleksandrowicz *et al.*, 2021).

Iron Uptake System Genes

Iron acquisition efficiency is critical for APEC survival within the host. Aerobactin and iron uptake systems (e.g., SitABCD and IutA) are important virulence determinants (Gao *et al.*, 2012). The studies have uncovered the regulatory networks that govern iron absorption systems, emphasizing their involvement in APEC pathogenesis, (Rodriguez-Siek *et al.*, 2005; Gao *et al.*, 2012; Niu *et al.*, 2022). APEC has been found to harbour multiple genes that encode the iron uptake and transport systems, including *iucCD*, *iutA*, *aerJ* (aerobactin), *iroBCDEN* (salmochelin), *fyuA* (yersiniabactin), *sitABCD*, *mntH* (iron and manganese transporter), *irp2* (iron repressible protein), *feoB* (ferrous ion transporter), *fepC* (ferric enterobactin transporter), *ireA* (iron-regulated virulence gene), *eitABCD* (putative iron transporter), *chuA* (outer membrane hemin receptor), and *bfr* (bacterioferritin) (Kathayat *et al.*, 2021).

Toxin-Encoding Genes

As discussed earlier the genes responsible for toxins production by APEC contribute to tissue injury. The cytotoxic necrotizing factor 1 (CNF1) and hemolysin are key contributors to host cell damage (Garcia *et al.*, 2013). Advances in molecular biology have revealed the complicated regulatory systems that govern toxin gene expression, providing prospective treatment targets (Kathayat *et al.*, 2021).

Capsule-Associated Genes

APEC is protected from host immunological responses by capsular polysaccharides. APEC's ability to avoid host defenses is influenced by genes involved in capsule formation, such as *kii* (K capsule encoding

genes), *kps*, and *traT*. The diversity of capsule forms among APEC strains correlates with specific types of pathogenicity variations (Ewers *et al.*, 2003; Mellata *et al.*, 2003; Ewers *et al.*, 2007).

Invasin-Encoding Genes

APEC strains have invasins that aid them in invading host cells. Hejair *et al.*, (2017) reported that genes that encode for outer membrane proteins facilitate APEC survival and proliferate within host macrophages. Molecular research is continuing to reveal the complex interplay between invasins and host cellular processes. The APEC have been found to harbour many genes encoding invasins, *ibeA* (also known as *ibe10*), *ibeB* (invasion protein), *tia* (toxigenic invasion locus), and *gimB* (genetic island linked with newborn meningitis). The *ibeR* operon regulator plays a role in invasion, resistance to environmental and serum stressors, and virulence gene expression. Likewise, *ychO*, a suspected invasin gene, contributes to biofilm formation, adhesion, invasion, motility, and the expression of genes involved in metabolism and membrane proteins (Kathayat *et al.*, 2021).

Type VI Secretion System (T6SS)

The Type VI Secretion System (T6SS) is a new virulence factor in APEC, implicated in bacterial competition and host interactions ((Journet and Cascales 2016). T6SS plays a crucial role in APEC pathogenicity and its potential as a target for therapeutic interventions (Ma *et al.*, 2014; Yin *et al.*, 2021). Two potential T6SS loci, such as T6SS and *vgrG*, have been shown to exist in the genome of some avian pathogenic *Escherichia coli* (APEC) strains. Of these two loci, VgrG-T6SS2 was exclusively accountable for cerebral infection, while T6SS1 contributed to the spread of APEC in systemic infection (Ma *et al.*, 2014).

ANTIBIOTIC RESISTANCE

Antibiotic resistance in APEC is an increasing concern that threatens poultry health and food safety. Kathayat *et al.*, (2021) reported that APEC strains possess several resistance genes, which contribute to the formation of multidrug-resistant isolates. Poole (2005) reported that Efflux-mediated antimicrobial resistance in APEC.

Common Antibiotic Resistances in APEC: APEC strains frequently display resistance to numerous types of antibiotics, including beta-lactams, tetracyclines, aminoglycosides, fluoroquinolones, sulfonamides, vancomycin, erythromycin, cefotaxime, Amoxicillin-Clavulanic acid and Ampicillin (Johnson *et al.*, 2007; Krishnegowda *et al.*, 2022; Özavcı *et al.*, 2022). This broad-spectrum resistance can make treating APEC infections in chickens more difficult.

Beta-Lactam Resistance

Resistance to beta-lactam antibiotics like penicillins and cephalosporins is common in APEC. Resistance is by production of the beta-lactamases, which hydrolyze the beta-lactam ring and render antibiotics ineffective (Rodríguez-Siek *et al.*, 2005).

Tetracycline Resistance

APEC strains frequently have genes that provide resistance to tetracycline, a common antibiotic used in chicken farming. Tetracycline resistance genes like *tet(A)* and *tet(B)* are frequently found in APEC isolates (Guabiraba and Schouler 2015; Aberkane *et al.*, 2023).

Fluoroquinolone Resistance

Fluoroquinolones are crucial antibiotics in both human and veterinary medicine. Mutations in the quinolone resistance-determining regions (QRDRs) of DNA gyrase and topoisomerase IV have been found in APEC strains, resulting in reduced susceptibility to fluoroquinolones (Lupo *et al.*, 2012).

Aminoglycoside Resistance

Resistance to aminoglycoside antibiotics is mediated by genes encoding enzymes that alter or inactivate the antibiotics, preventing them from binding to the bacterial ribosome (Nowaczek *et al.*, 2021).

Sulfonamide Resistance

Sulfonamide resistance in APEC is frequently related to the presence of *sul* genes, which encode enzymes involved in the formation of dihydropteroate, the target of sulfonamide antibiotics (Aberkane *et al.*, 2023).

Extended-Spectrum Beta-Lactamase (ESBL) Production

ESBLs, enzymes that impart resistance to extended-spectrum cephalosporins and other beta-lactam antibiotics, are produced by some APEC strains (Olsen *et al.*, 2014; Aberkane *et al.*, 2023; Chowdary *et al.*, 2023). CTX-M-type enzymes are common ESBLs in APEC (Olsen *et al.*, 2014).

Understanding the antibiotic resistance profile of APEC is critical for establishing prudent antibiotic use in poultry farming and reducing the potential of resistant strain transmission to humans. Continuous surveillance and research efforts are essential to monitor and address the evolving antibiotic resistance in APEC.

CLINICAL MANIFESTATIONS

APEC is associated with a wide spectrum of clinical symptoms in chickens, resulting in major economic losses and management issues. The diverse clinical consequences found during APEC infections encompass respiratory distress, systemic infections, and internal organ lesions, with a focus on the impact on flock health and productivity.

Respiratory Distress

APEC infections frequently cause respiratory discomfort, with clinical indications such as coughing, sneezing, nasal discharge, and swollen sinuses (Dziva and Stevens 2008). In affected birds, these respiratory symptoms contribute to decreased feed conversion efficiency and altered respiratory function.

Systemic Infections

Krishnegowda *et al.*, (2020) and Krishnegowda *et al.*, (2022), reported that APEC can cause systemic infections, leading to septicemia and affecting various organs. Clinical symptoms of systemic involvement include depression, lethargy, and huddling, which often leads to higher mortality rates in infected flocks. The economic impact on the chicken industry is exacerbated by APEC's systemic spread.

Pericarditis and Osteomyelitis

APEC infections can cause pericarditis (inflammation of the pericardial sac surrounding the heart) and osteomyelitis (bone lesions) (Ewers *et al.*, 2003; Ewers *et al.*, 2007; Braga *et al.*, 2016). These manifestations contribute to cardiac functional impairment, lameness, restricted movement, and skeletal abnormalities, negatively impacting chicken health and productivity.

Colibacillosis

Colibacillosis, the collective term for diseases caused by pathogenic *E. coli*, has a wide range of clinical symptoms. This comprises cellulitis, salpingitis, and peritonitis, with clinical symptoms such as swollen wattles, abdominal distension, and egg-laying anomalies (Newman *et al.*, 2018). Colibacillosis has a substantial impact on egg production and quality.

Lesions in Internal Organs

APEC infections are linked to internal organ lesions, particularly in the respiratory and other visceral organs. These lesions contribute to the reported clinical symptoms and indicate the pathogen's ability to colonize and penetrate host tissues (Gao *et al.*, 2012).

Studies have provided insight into the clinical diversity of APEC infections, emphasizing the necessity of understanding strain-specific differences in virulence and host responses (Ali *et al.*, 2020; Aleksandrowicz *et al.*, 2021). Finally, the clinical symptoms of APEC infections in chickens are numerous, including respiratory, systemic, and reproductive problems. A thorough understanding of these manifestations is critical for earlier diagnosis and the development of effective solutions to limit APEC's influence on poultry health.

GROSS PATHOLOGY

Infections with APEC in chickens cause different gross pathological alterations that reflect the disease's multi-organ involvement and severity. Thickened air

sacs with deposition of fibrinous exudates; congestion, oedema, hemorrhages with fibrin deposition on the pleural surface of pneumonic lungs; pericarditis along fibrinous exudates, epicardial congestion and hemorrhages in heart; enlargement of the liver with congestion, hemorrhages, and perihepatitis along with fibrinous deposits of varying thickness; swollen spleen with congestion, hemorrhages, and fibrinous exudates on the surface; kidneys with congestion and hemorrhages; peritonitis are the common macroscopic lesions associated with APEC infection in chicken (Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022).

Air Sacculitis and Pericarditis

APEC infections frequently result in airsacculitis, which is characterized by thickened sac and the deposition of caseous or fibrinous exudate in the air sacs. Pericarditis is prevalent and manifests as a thickened pericardial sac with fibrinous deposits (Dziva and Stevens 2008; Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022). These disorders aggravate respiratory discomfort and impair heart function.

Swollen and Congested Organs

The visceral organs particularly the liver and spleen exhibit enlargement, congestion, haemorrhages, and fibrinous deposits on their surface (Fig 1). Hepatomegaly and splenomegaly are evident, reflecting the systemic spread of APEC and the associated septicemia (Ewers *et al.*, 2003; Ewers *et al.*, 2007; Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022). The liver may also develop localized necrotic lesions, limiting its function even further.

Lesions in Reproductive Organs

Infections with APEC can cause lesions in the reproductive organs, particularly the oviduct. Salpingitis, characterized by oviduct inflammation and purulent discharge, is a common symptom (Van Goor *et al.*, 2020; Mehat *et al.*, 2021). These lesions contribute to decreased

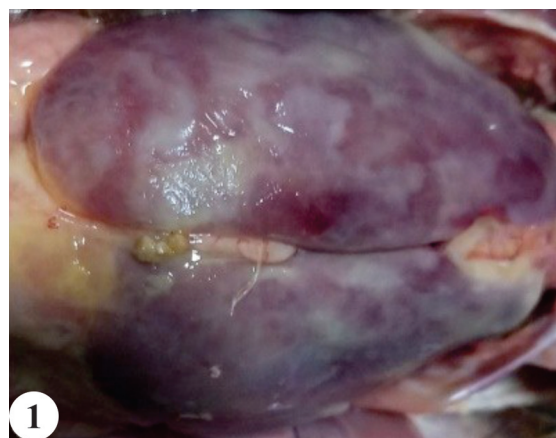


Fig 1. The yellow creamy fibrinous exudate on chicken liver indicative of perihepatitis and hepatitis due to APEC infection.

egg yield and egg quality.

Joint Involvement and Osteomyelitis

APEC infections can cause arthritis, with visible signs of joint swelling and inflammation. Concurrently, osteomyelitis, characterized by bone lesions, particularly in the long bones of the legs, contributes to lameness and skeletal abnormalities (Braga *et al.*, 2016).

Cellulitis and Subcutaneous Lesions

The gross examination frequently reveals subcutaneous lesions and cellulitis, as well as swollen and discoloured skin in affected areas (De Oliveira *et al.*, 2020). These lesions may be accompanied by the presence of gas within the tissues, which contributes to the typical bad odour associated with colibacillosis.

The studies highlight the necessity of knowing the gross pathology of APEC infections to aid in correct diagnosis and intervention efforts (Ewers *et al.*, 2003; Ewers *et al.*, 2007; De Oliveira *et al.*, 2020; Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022). In summary, APEC infections in chickens are characterized by a variety of abnormalities affecting multiple organs. These gross pathological changes indicate systemic involvement in APEC infections, emphasising the significance of a complete assessment for optimal disease management.

HISTOPATHOLOGY

Infections with APEC in chickens are associated with different microscopic lesions, offering significant insights into disease pathophysiology. Various microscopic lesions characteristic of APEC infections has been reported in organs, ranging from acute inflammatory reactions such as vascular changes to chronic lesions such as granulomatous inflammation and fibrosis. Fibrinous exudation, indicative of severe acute vascular damage. It is considered a very characteristic finding in septicemia caused by APEC. Also, other characteristic lesions such as fibrin deposits in the sinusoids of the liver; hyperplasia of reticular cells surrounding the penicilliform capillaries, and fibrinoid necrosis in the spleen have been recorded. Chronic granulomatous lesions with bizarre giant cells along with intralesional bacteria were reported from most of the organs in APEC infection (Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022).

Air Sac Lesions

Air sac in APEC infected chickens exhibit lesions in the air sacs such as fibrinopurulent inflammation and heterophilic infiltration (Fig 2) (Dziva and Stevens 2008; Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022). The diversity of APEC strains recovered from broilers, emphasizes the importance of understanding strain-specific changes in air sac lesions.

Lung lesions

In the lungs, more often, typical caseogranulomatous inflammation characterized by pinkish caseous necrotic debris in para bronchi surrounded by a zone of giant cells and also an admixture of inflammatory cells has been encountered (Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022) (Fig 3). In severe cases of pneumonia, the disruption of atria and even inflammation has spread to the interstitium. More severe pneumonic lungs, mild fibrosis in the lung parenchyma, and pleuropneumonia (characterized by pleuritis with fibrinoheterophilic exudates) changes have also been observed (Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022).

Pericarditis and Myocarditis

APEC infections usually cause pericarditis, with histological evidence of fibrinous inflammation with different inflammatory cellular infiltration into the pericardial sac (Fig 4). In severe cases, involvement of myocardium, characterized by inflammatory alterations in the heart muscle, may also be evident (Ewers *et al.*, 2003; Ewers *et al.*, 2007; Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022).

Hepatic and Splenic Lesions

The liver of APEC-infected chicken exhibits multifocal necrotic lesions and inflammatory infiltrates (Fig 5), indicating that APEC has progressed throughout the body. Splenic lesions may include congestion, haemorrhage, and necrosis as a result of APEC's effect on the reticuloendothelial system (Gao *et al.*, 2012; Krishnegowda *et al.*, 2020; Krishnegowda *et al.*, 2022).

Reproductive Organ Pathology

Infections with APEC can cause histological abnormalities in the reproductive organs, especially the oviduct. Salpingitis is a common occurrence in the oviduct mucosa, characterized by inflammation, epithelial degradation, and heterophil infiltration (Van Goor *et al.*, 2020; Mehat *et al.*, 2021). These abnormalities contribute to lower egg production and poor egg quality.

Joint and Bone Involvement

In APEC-infected chickens, arthritis and osteomyelitis are prevalent, with microscopic changes such as synovial inflammation, joint effusion, and bone lesions (Ewers *et al.*, 2003; Ewers *et al.*, 2007). The bone abnormalities are caused by this tissue damage.

Cellulitis and Subcutaneous Lesions

Histopathological investigation of APEC-infected chicken subcutaneous lesions showed inflammation, cellular infiltration, oedema, and necrosis of the subcutaneous tissues (De Oliveira *et al.*, 2020).

Recent research, including studies by Krishnegowda *et al.*, (2020) and Krishnegowda *et al.*, (2022), emphasizes the importance of histopathological investigations in

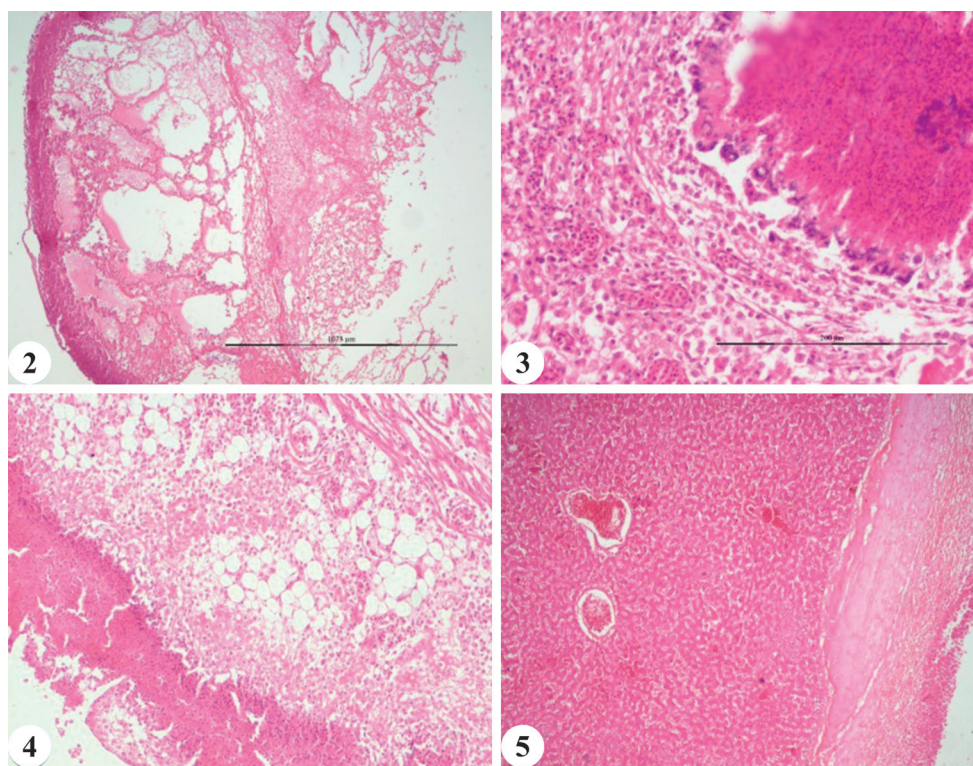


Fig 2. The air sac of a broiler chicken showing disruption of the entire epithelial lining due to necrosis and a marked increase in air sac thickness due to inflammation, inflammatory cells (lymphocytic and heterophilic), and fibrin. Extensive oedema is present in the lumen, evident by the accumulation of uniform eosinophilic exudates combined with fibrins (H&E x40); **Fig 3.** The lung of a broiler chicken reveals caseogranulomatous pneumonia, characterized by caseous necrotic debris in the parabronchi, rimmed by giant cells and the presence of intralesional bacteria (inside necrotic debris). The interlobular septa thickened, oedematous, and infiltrated with fibrinoheterophilic exudates (H&E x200); **Fig 4.** The heart of a broiler chicken, showing pericarditis and epicarditis characterized caseogranulomatous reaction with fibrinoheterophilic exudates. The mild inflammatory cellular infiltration into the myocardium. (H&E x40, b, x200); **Fig 5.** Liver of chicken with perihepatitis, accumulation of caseous necrotic mass and fibrin on the liver surface along with infiltration of heterophils into the parenchyma.

understanding the dynamic nature of APEC infections and the host response. In conclusion, the microscopic lesions in APEC-infected chickens are heterogeneous and reflect the disease's systemic nature. Histopathological investigation is still an important technique for making a good diagnosis and understanding the complicated interplay between APEC and the host.

DIAGNOSIS

There is an array of diagnostic methods for detecting APEC infections, from traditional culture techniques to modern molecular and serological approaches available. The significance of precise and timely disease diagnosis is imperative for optimal disease management and therapeutic intervention. Recent advancements in diagnostic tests have improved the sensitivity and specificity.

In this section, we explore some of the following contemporary diagnostic methods for a comprehensive understanding.

Polymerase Chain Reaction (PCR) Assays

Because of their great sensitivity and specificity, PCR-based technologies such as conventional PCR and quantitative real-time PCR (qPCR) have become essential for APEC detection. These assays, target virulence genes such as *iroN*, *iutA*, *iss* etc., (Krishnegowda *et al.*, 2022) and allow rapid and accurate detection of APEC strains (Schouler *et al.*, 2012).

Multiplex PCR

Multiplex PCR detects numerous virulence genes at the same time, providing a more thorough profile of APEC strains (Johnson *et al.*, 2006; Johnson *et al.*, 2008a, 2008b; Krishnegowda *et al.*, 2022). This approach serves to identify pathotypes and determine the possible severity of infection (Mora *et al.*, 2014).

Whole Genome Sequencing (WGS)

WGS has evolved as an effective method for APEC characterization, allowing for detailed genomic analysis (Johnson *et al.*, 2007; Chen *et al.*, 2021). It contributes to a better knowledge of APEC epidemiology by providing information on virulence factors, antibiotic resistance, and strain diversity (Dziva and Stevens 2008).

Enzyme-Linked Immunosorbent Assay (ELISA)

A serological method for diagnosing APEC infections is provided by ELISA-based techniques that identify particular APEC antigens or antibodies in chicken serum. These tests help with large-scale surveillance investigations and seroepidemiology (Leitner *et al.*, 1990; Schouler *et al.*, 2012; Wilczyński *et al.*, 2022).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS has shown potential for identifying bacterial isolates, including APEC, quickly and accurately. This technique uses spectral analysis to identify microorganisms, making it a useful tool for routine diagnostics (Bej *et al.*, 2009).

Loop-Mediated Isothermal Amplification (LAMP)

LAMP is an isothermal nucleic acid amplification technology that is quick and easy to use. LAMP assays have proven good sensitivity and promise for on-site testing when used for APEC detection (Kogovšek *et al.*, 2019).

Recent past investigations, such as those by Ewers *et al.*, 2003; Ewers *et al.*, (2007), and Krishnegowda *et al.*, (2022), illustrate the variety of diagnostic approaches and emphasize the need for continuous refinement and validation. To summarise, contemporary APEC diagnostic tests in chickens include molecular, serological, and mass spectrometry-based methods. These advancements improve our ability to recognize APEC quickly and precisely, allowing for immediate intervention and control measures.

PREVENTION AND CONTROL

APEC is a serious threat to poultry health and production, resulting in financial losses and issues for the poultry industry. A successful APEC management strategy combines preventive measures and control tactics.

Here we describe current APEC infection prevention and control measures in chickens. Vaccination, biosecurity measures, antimicrobial stewardship, and the possible significance of probiotics in the prevention of APEC-associated illnesses are covered. Also discusses the difficulties and opportunities in developing novel control systems.

Biosecurity Measures

The use of strong biosecurity measures is critical in limiting the introduction and spread of APEC within poultry facilities. Strict hygiene standards, restricted access, and proper disinfection techniques all help to reduce the danger of APEC transmission (Joseph *et al.*, 2023).

Vaccination Strategies

Vaccination is critical in preventing APEC infections. Several vaccines have been developed that target certain APEC serotypes or virulence factors. These vaccinations are designed to minimize the severity of infections, fatality rates, and APEC strain shedding (Soleymani *et al.*, 2020; Christensen *et al.*, 2021; Dai *et al.*, 2023).

Antimicrobial Stewardship

Antibiotic stewardship is critical in reducing APEC infections and preventing the establishment of antibiotic-resistant strains. The prudent use of antimicrobials, adherence to withdrawal periods, and the implementation of antimicrobial stewardship programmes all contribute to the reduction of resistance development (Rodríguez-Siek *et al.*, 2005).

Genetic Selection for Resistance

Breeding programmes that combine genetic selection for APEC resistance have shown promise in improving poultry innate immunity. Selecting features like better mucosal immunity and resistance to respiratory infections can help reduce avian susceptibility to APEC (Johnson *et al.*, 2007).

Nutritional Interventions

A proper diet is critical for sustaining poultry's general health and immunological function. Nutritional treatments, such as the use of feed additives like probiotics, prebiotics, and organic acids, have been investigated as ways to boost the immune response and lessen the impact of APEC infections (Deepthi *et al.*, 2017; Kathayat *et al.*, 2021). Wang *et al.*, (2023) reported that polysaccharides from *Pinus massoniana* Lamb. pollen (PPPSt) has anti-inflammatory properties and can reduce myocardial injury caused by bacterial septicemia.

Management Practices

Optimizing management methods like adequate ventilation, stocking density, and litter control contribute to stress reduction and the prevention of APEC proliferation. Well-managed poultry houses make an environment that is less conducive to the development of respiratory diseases (Johnson *et al.*, 2007).

Research and Surveillance

It is critical to do ongoing research on APEC epidemiology, virulence factors, and host-pathogen interactions to develop effective prevention and control techniques. Surveillance programmes aid in the monitoring of APEC prevalence, antibiotic resistance patterns, and the introduction of novel strains, allowing for early responses (Selvarajan *et al.*, 2022; Yousef *et al.*, 2023).

Recent studies, emphasize the necessity of integrated tactics and ongoing research initiatives (Selvarajan *et al.*, 2022) to overcome *E. coli* infection. To summarise, preventing and controlling APEC infections in chickens

requires a comprehensive and integrated approach that includes biosecurity, immunization, antimicrobial stewardship, genetic selection, nutritional interventions, and good management methods.

ONE HEALTH PERSPECTIVE

In this section, emphasis is given to exploring the interconnectedness of animal, human, and environmental health concerning APEC infections.

APEC is a complex dilemma at the crossroads of human, animal, and environmental health that necessitates a One Health strategy for full understanding and effective control. This part investigates APEC's One Health viewpoint, using contemporary sources to provide current perspectives.

Transmission Dynamics

APEC can serve as a reservoir of antibiotic resistance genes and virulence factors, potentially affecting human health through direct transmission or the transfer of genetic elements to human-pathogenic *E. coli* strains (Johnson *et al.*, 2007). Understanding the mechanisms of transmission across species barriers is critical for minimizing zoonotic transmission risk.

Antimicrobial Resistance

Antibiotic resistance in APEC strains is common in both veterinary and human medicine. The spread of antibiotic resistance from poultry to humans or the environment, emphasizes the interconnection of health across sectors (Rodríguez-Siek *et al.*, 2005). This emphasizes the importance of coordinating efforts to combat antibiotic resistance.

Environmental Contamination

Through faecal shedding, APEC can contaminate the environment, aiding in the spread of antibiotic-resistant bacteria and resistance genes. This environmental contamination could be a cause of human exposure and infection (Selvarajan *et al.*, 2022). Environmental contamination must be monitored and mitigated as part of a One Health plan.

Impact on Poultry Production

Infections with APEC cause economic losses and decreased productivity in poultry. The interconnectedness of animal health and production highlights the importance of addressing APEC infections for both animal welfare and sustainable food production, (Dziva and Stevens 2008).

Surveillance and Early Detection

One Health surveillance systems are crucial in detecting and monitoring APEC early. Integrating human, animal, and environmental data allows for a more comprehensive understanding of APEC epidemiology and allows for more prompt intervention

methods (Yousef *et al.*, 2023).

Public Health Awareness

Educating healthcare professionals, veterinarians, farmers, and the general public about the hazards associated with APEC and the importance of responsible antibiotic use promotes a collaborative One Health approach. Awareness efforts help to slow the spread of APEC and the associated antibiotic resistance (Johnson *et al.*, 2007).

Researchers have emphasized the connectivity of APEC across sectors and the significance of coordinated research and surveillance efforts in a One Health framework, (Ewers *et al.*, 2003; Ewers *et al.*, 2007). Finally, APEC shows the complex interaction that exists between human, animal, and environmental health. A One Health approach that highlights the interconnection of different health sectors is critical for addressing the multifaceted issues faced by APEC.

FUTURE PERSPECTIVES

The changing environment of microbial pathogens, particularly APEC, needs a forward-thinking approach to foresee issues and identify viable research and intervention pathways. Taking into account emerging trends, technological breakthroughs, and research agendas, the following aspects may be prioritized.

Genomic Surveillance and Epidemiology

The incorporation of new genomic technologies, such as whole-genome sequencing (WGS), will be critical in improving our understanding of APEC epidemiology. Genomic monitoring can reveal information about strain diversity, evolution, and the establishment of new virulence factors or resistance mechanisms (Mageiros *et al.*, 2021; Kravik *et al.*, 2023).

Characterization of Virulence Factors

Continued study into the molecular mechanisms of APEC virulence is required for the development of tailored intervention techniques. Understanding how specific virulence variables influence host-pathogen interactions can help guide the development of new treatments or vaccines (Dziva and Stevens 2008; Kathayat *et al.*, 2021; Ovi *et al.*, 2023).

Host-Pathogen Interactions

In-depth research of the host immune response to APEC infections will be essential for creating immunomodulatory therapies and improving host resistance. Studying the molecular basis of host-pathogen interactions can reveal useful insights into APEC's immune evasion mechanisms (Dziva and Stevens 2008).

Antibiotic Alternatives and Antimicrobial Stewardship

Given the widespread worry about antibiotic resistance, future research should concentrate on creating

antibiotic alternatives for APEC management. Probiotics, phage treatment, and other antimicrobial medicines may provide long-term options, and antimicrobial stewardship programmes will be critical in minimising resistance development (Fancher *et al.*, 2020 Bastidas-Caldes *et al.*, 2022; Wang *et al.*, 2023).

One Health Integration

It will be critical to strengthen the integration of One Health ideas in APEC research. This includes efforts by human and veterinary healthcare experts, environmental scientists, and politicians to address APEC's interdependence across domains (Johnson *et al.*, 2007).

Vaccine Development and Immunization Strategies

Vaccine advancements, such as subunit vaccines, recombinant vaccines, and novel adjuvants, offer prospective pathways for APEC immunisation. Future research should concentrate on producing vaccines that give broad-spectrum protection against a wide range of APEC strains (Soleymani *et al.*, 2020; Kathayat *et al.*, 2021; Ovi *et al.*, 2023).

Climate Change and Ecological Factors

It is critical to consider the impact of climate change on chicken production as well as the potential influence of ecological factors on APEC prevalence. Adapting preventive tactics will require research into how environmental changes affect the epidemiology and pathogenicity of APEC (Selvarajan *et al.*, 2022; Li *et al.*, 2022).

Precision Medicine Approaches

Precision medicine techniques tailored to the individual characteristics of APEC strains could transform treatment strategies. Understanding strain-specific virulence variables and resistance profiles can help guide therapy selection (Dziva and Stevens 2008).

Global Surveillance Networks

APEC's worldwide surveillance networks will be established and improved, allowing for real-time monitoring of new strains, antimicrobial resistance patterns, and epidemiological trends. Early detection and reaction to possible epidemics can be aided by collaborative efforts.

Public Awareness and Education

Raising awareness about APEC, its implications, and prevention actions among poultry producers, veterinarians, healthcare professionals, and the general population is critical. Training programmes can help to increase biosecurity and responsible antibiotic usage.

A comprehensive and collaborative strategy will be required to address the complexities of this disease and its impact on poultry and public health as we navigate the future of APEC research and control.

CONCLUSIONS

In conclusion, the multifaceted challenges posed by APEC necessitate ongoing research, collaboration, and a proactive approach. By embracing a comprehensive understanding of APEC, from its molecular intricacies to its ecological implications, we can strive towards effective strategies for the prevention, control, and sustainable management of APEC infections. This review serves as a stepping stone for researchers, veterinarians, and policymakers to engage in a concerted effort towards safeguarding poultry health, ensuring food safety, and addressing the broader implications of APEC within the One Health framework.

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Avian coccidiosis

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ABSTRACT

Coccidiosis is an economically significant disease of commercial and backyard chicken in India. It is caused by protozoan parasites of the genus *Eimeria*, which are a group of monoxenous obligate intracellular parasites. Seven species of this genus can affect chickens (*Gallus gallus*), each with different pathogenic characteristics and targeting a specific intestinal location. *Eimeria* alters the function of the intestinal tract, causing deficiencies in the absorption of nutrients and lowering productive performance, leading to economic losses. The concern of resistance towards major anticoccidials has provided a way forward to vaccine research and development. For prophylaxis of chicken coccidiosis, live virulent, attenuated, and recombinant vaccines have been extensively trialed and commercialized. The cost of production and achieving robust immune responses in birds are major challenges for commercial vaccine production. Some of the nutritional interventions that have shown potential for improving gut health while reducing overall disease include the use of probiotics, prebiotics, organic acids, essential oils, and natural phytochemical extracts in poultry diets. This review article describes recent advancement in diagnosis and prevention and control of avian coccidiosis in chicken.

Keywords: Coccidiosis, chicken, diagnosis, prevention, control

INTRODUCTION

The poultry industry is one of the fastest growing sub-sectors of agriculture that contributes to global nutrition and thus a major driving force of the economy (Mottet and Tempio, 2017). However, chickens are also host to many deadly diseases which hamper productivity and compromise welfare resulting in high mortality. Among many diseases that affect chickens globally, coccidiosis is associated with high mortality in poultry industry (Blake and Tomley, 2014). In India, total poultry population is 851.81 million in 2019 and has exported 320,240.46 MT of poultry products to the world for the worth of Rs.529.81 Crores (71.04 USD Millions) during the year 2021-22 (APEDA). The global economic losses caused by coccidia infection reached 10.4 billion pounds every year (Blake *et al.*, 2020). Coccidiosis is highly prevalent in India especially in the monsoon season. The prevalence of coccidiosis in broilers may range between 5% and 70% depending on many factors, such as management, presence of vectors, *Alphitobius*, high temperature and humidity (Du and Hu, 2004). It is estimated that practically all farms are affected by this disease at some time of the year, either in the form of clinical or subclinical coccidiosis. The emerging poultry industry of India incurs a huge economic loss due to mortality, increased feed conversion ratios, reduced weight gains, drop in production, lower reproductive performance, cost of treatments, continuous *Eimeria* oocyst shedding, and increased susceptibility towards secondary bacterial infections (Adhikari *et al.*, 2020). Prophylaxis of coccidiosis is of utmost importance to promote substantial growth in chicken industry and to protect the sources of financial income. In India, it is not possible to maintain the current level of chicken production without a comprehensive anticoccidial management program. Therefore, all the commercial poultry farmers use antiparasitic drugs as feed additives. Phytochemical or herbal medicines seem to be a promising strategy for preventing coccidiosis, in accordance with the “anticoccidial chemical-free” standards (Fatoba and Adeleke, 2018).

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ETIOLOGY

Coccidiosis is primarily caused by intracellular protozoan *Eimeria*. The *Eimeria* are obligate, intracellular, apicomplexan parasites belonging to *Eimeridae* family and genus *Eimeria* (El-Shazly *et al.*, 2020). So far in chicken 7 *Eimeria* species have been recognized as responsible for intestinal disease. These include *Eimeria* (*E.*) *praecox*, *E. acervulina*, *E. mitis*, *E. brunetti*, *E. tenella*, *E. maxima*, and *E. necatrix*. Among all species, *E. maxima*, *E. tenella*, *E. acervulina*, and *E. necatrix* have highest clinical and sub-clinical disease burden, making them economically significant among *Eimeria* species of poultry (Hinsu *et al.*, 2018). Different species of coccidia have similar host infection specificities, but different parasitic sites and virulence. Cecum is the main

infection site of *E. tenella* (Guo *et al.*, 2013) and the middle part of the small intestine is the main infection site of *E. necatrix* and *E. maxima* (Reperant *et al.*, 2021) while duodenum is the site of *E. acervulina*. Concurrent infection with 2 or more species of coccidia is common. The morphology of coccidia oocysts is similar for most *Eimeria* species. They are ellipsoidal or circular shaped with a thick cell wall and sporocysts. Majority of *Eimeria* oocysts have ovoid shape. *E. maxima* ($30.5 \times 20.7\mu\text{m}$) is the largest while *E. mitis* ($15.6 \times 14.2\mu\text{m}$) is the smallest as compared to other species of *Eimeria*. *E. tenella*, *E. maxima*, *E. acervulina*, and *E. burnetti* are ovoid while *E. necatrix* is oblong (Clark and Blake, 2012).

Transmission and Vectors

Ingestion of sporulated oocysts is the only natural method of transmission of coccidiosis. Once infected, chickens may shed oocysts in the feces for several days or weeks. The oocysts in feces become infective through the process of sporulation in about 2 days. Susceptible birds in the same flock may ingest the oocysts through litter-pecking or the contamination of feed or water. No natural intermediate hosts exist for the *Eimeria* spp., but oocysts can be spread mechanically by a multitude of vectors, including insects, contaminated equipment, wild birds, and dust. Oocysts generally are considered resistant to environmental extremes and to disinfectants,

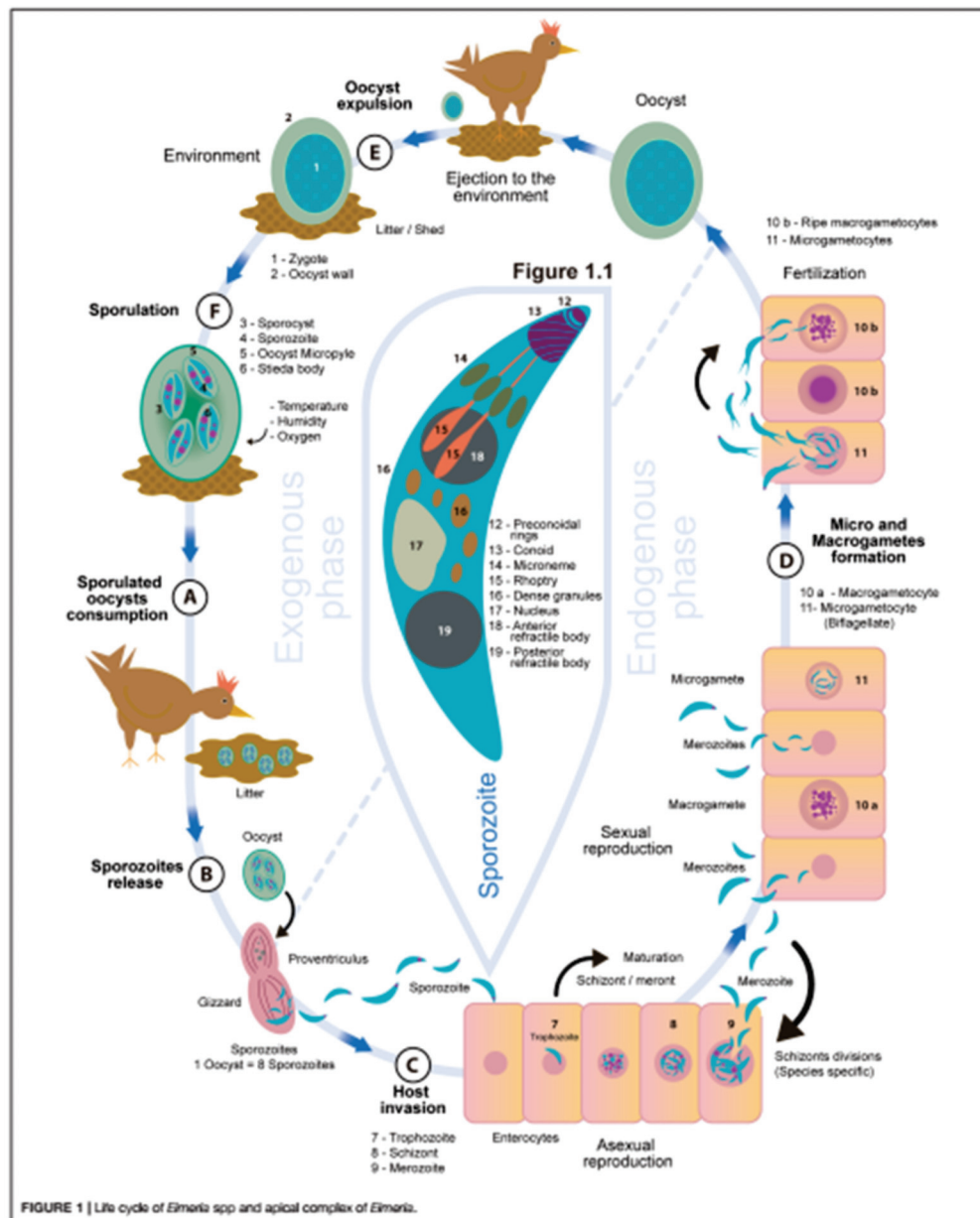


Fig 1. Life cycle of *Eimeria* spp.

although survival time varies with conditions. Oocysts may survive for many weeks in soil, but survival in poultry litter is limited to a few days because of the heat and ammonia released by composting and the action of molds and bacteria. The darkling beetle (*Alphitobius diaperinus*), common in broiler litter, is a mechanical carrier of oocysts (Shirley *et al.*, 2005).

Life Cycle

Eimeria have a direct life cycle, characterized by high tissue and host specificity, involving stages of asexual and sexual multiplication, with three development phases: the formation of schizogony (agamogony/merogony), gametogony (gamete formation for sexual reproduction), and sporogony (Cervantes *et al.*, 2020). As shown in Fig 1, transmission occurs via fecal-oral route and infection begins with the ingestion of sporulated oocysts. The oocyst, a thick-walled zygote shed in fecal matter by the infected host. The process of sporulation begins immediately, under the optimal conditions of temperature, moisture, and oxygenation, oocysts sporulate and become infective within 24–72 hours (Haug *et al.*, 2008). When sporulated oocysts are ingested, wall of the oocyst is crushed in the ventriculus, releasing the sporocysts. The sporozoites emerge from the sporocysts by the action of chymotrypsin and bile salts in the small intestine (Chapman *et al.*, 1978). The sporozoites contained within each sporocyst are released into the intestinal lumen by the process called excystation (Kheysin, 1972). The sporozoites invade the enterocytes, changing into trophozoites and starting a parasitic feeding period that lasts ~12–48 h. The parasitophorous vacuole is formed, the trophozoite begins to enlarge, and the parasite nucleus performs multiple asexual divisions forming the schizont or meront, which is full of merozoites. Approximately 3 days post infection, the mature schizont ruptures and releases the merozoites, which are fusiform and have an apical complex that allows them to move and infect intestinal epithelial cells to form additional schizont generations that reproduce asexually (Nabian *et al.*, 2018).

When the asexual reproduction phase is complete, the sexual reproduction stage or gametogony begins, occurring in three events. The first is gametocytogenesis, in which gametocytes are produced from merozoites. Second, during gametogenesis, haploid micro and macrogametes are differentiated from the gametocytes. Finally, macrogametocytes are fertilized by microgametocytes producing diploid zygotes, at which point sexual reproduction is completed; meiosis proceeds, inside the protective oocyst wall, followed by mitosis to produce the infectious sporozoites. The macrogametocyte grows quickly and forms a single macrogamete while the microgametocyte matures, breaks up and releases many small biflagellate microgametes that are a vehicle

to deliver DNA (Walker *et al.*, 2013). Once the oocyst is excreted from the animal in the feces, the third phase of the cycle, sporulation, takes place. If environmental conditions are adequate, the diploid oocyst initiates sporogony formation. With each species, the reproductive potential from a single ingested oocyst is constant. The entire process takes 4–6 days, depending on species, although oocysts may be shed for several days after patency is reached. In some species (*E. tenella*, *E. necatrix*), the maximum tissue damage may occur when second-generation schizonts rupture to release merozoites. Other species may have small scattered schizonts, which cause little damage, but the gametocytes may elicit a strong reaction with cellular infiltration results in thickening of mucosa of affected part of intestine.

CLINICAL SIGNS

Degree of infection and the clinical signs of coccidiosis are influenced by multiple factors including the species of *Eimeria*, the infective dose, host-parasite interactions and environmental conditions of the poultry house. Several studies have shown that there is an optimal dose, such that the reproductive potential of the parasite is met, and they efficiently replicate within the epithelial cells. Very high doses of ingestion can cause “crowding effect,” interrupting the continuity of the parasite’s life cycle, while still generating intestinal damage (Williams, 2005). The host is a pivotal factor of *Eimeria* infections in poultry. Some *Eimerian* parasites are highly immunogenic in chickens, and primary infections can stimulate protective immunity to subsequent challenge by the homologous parasite (Lillehoj *et al.*, 2000).

The disease may be mild, resulting from the ingestion of a few sporulated oocysts, and may escape notice, or it may be severe as a result of ingestion of millions of sporulated oocysts. Infected birds present with ruffled feathers and signs of depression or drowsiness. Additionally, feed and water consumption are decreased, and the feces may be watery, whitish and occasionally bloody. This results in dehydration, impaired weight gain, and death in the absence of treatment (Adams *et al.*, 1996). The tissue damage and changes in intestinal tract permeability and function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis or salmonellosis (Adhikari *et al.*, 2020). Cecal coccidiosis may contribute to increased severity of the blackhead organism (*Histomonas meleagridis*) in chickens. Immunosuppressive diseases may act in concert with coccidiosis to produce a more severe disease. Marek’s disease may interfere with development of immunity to coccidiosis and infectious bursal disease and chicken infectious anemia may exacerbate coccidiosis, placing a heavier burden on anticoccidial drugs (Cervantes *et al.*, 2020).

DIAGNOSIS

Correct identification of *Eimeria* species is important for the diagnosis and control of the disease (Carvalho *et al.*, 2011). Classical methods for the evaluation of *Eimeria* infections include macroscopic diagnosis with observation of clinical signs in infected animals, the location and appearance of gross lesions during necropsy; and microscopic diagnosis, which focuses on evaluating the size and shape of oocysts (Conway and McKenzie, 2007). Pathogenicity and characteristics lesions of each *Eimeria* is given in table 1.

For the evaluation of gross lesions, a standardized intestinal lesion scoring technique is used, which is based on giving a score on a scale of zero to four, with the goal of obtaining a numeric classification of the gross lesions caused by each *Eimeria* species (Johnson and Reid, 1970). The changes visible in the walls of infected organs by oocysts of *E. acervulina*, *E. maxima* and *E. tenella* and respective lesion scoring is summarized in table 2. Generally, a set number of birds per flock are assessed (between 5 and 6) and the individual scores are added for all *Eimeria* spp. (Raman *et al.*, 2011).

Microscopic identification

Scrapings of the intestinal mucosa should be taken to evaluate the presence and shape of oocysts. Typically, the intestinal lesion score is complemented with counts of oocysts per gram (OPG) of feces or poultry litter through the McMaster technique (Carvalho *et al.*, 2011). Recently, the Mini-FLOTAC has been developed as a new method for qualitative and quantitative diagnosis of infections by helminths and protozoans in several mammal hosts. The Mini-FLOTAC is a reliable and precise method of quantification for this species with the detection

limit ranging between 100 and 500 oocysts per gram of excreta (OPG) (Bortoluzzi *et al.*, 2018). The Mini-FLOTAC technique is based on flotation principles with saturated solutions, using a device with two components, Fill-FLOTAC and reading chamber.

Molecular diagnosis

When greater diagnostic precision is needed, molecular diagnostics must be included (Hinsu *et al.*, 2018). In field conditions, *Eimeria* infections are often caused by more than one species, with similar pathological lesions, making diagnosis difficult. Polymerase chain reaction (PCR) based on the amplification of regions of the Internal Transcribed Space 1 (ITS1) of the ribosomal DNA now a widely used technique (Hamidinejat *et al.*, 2010; Tang *et al.*, 2018). The ITS is a piece of non-functional RNA located between structural ribosomal RNAs (rRNA) on a common precursor transcript. This region of the genome contains several segments used for *Eimeria* spp. identification, including the 5' external transcribed sequence (5'ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA, and finally the 3'ETS. Other molecular techniques Random Amplified Polymorphic DNA (RAPD) (Shirley *et al.*, 2005), Sequence Characterized Amplified Region (SCAR) markers (Fernandez *et al.*, 2004), quantitative PCR (Vrba *et al.*, 2010) and Loop-Mediated Isothermal Amplification (LAMP) (Shu-San *et al.*, 2019) have been used for diagnosis of *Eimeria*. Next Generation Sequencing techniques (NGS) are allowing for precise identification of emerging or region-specific *Eimeria* spp. as well as facilitating other avenues of research for control of coccidiosis (Hinsu *et al.*, 2018; Hauck *et al.*, 2019).

CONTROL MEASURES

Table 1. Pathogenicity and characteristics lesions of each *Eimeria* spp.

<i>Eimeria</i> species	Development Site	Pathogenicity	Lesion scoring
<i>E. acervulina</i>	Duodenum, Jejunum	++	Mucoid enteritis, causing loss of fluids, and poor absorption of nutrients Whitish spots on serous surface, hemorrhage streak and plaques on mucosal surface in dead birds
<i>E. praecox</i>	Duodenum, Jejunum	+	Mild mucoid enteritis. No lesion but mild hemorrhagic appearance on serous surface of duodenum
<i>E. mitis</i>	Ileum	+	Mild enteritis, causing loss of fluids and poor absorption of nutrients.
<i>E. necatrix</i>	Jejunum, Ileum, Cecum	+++	Thickening of the mucosa and intestinal lumen filled with liquid, blood and the remains of tissue Lesions in dead birds are observable as white and black sheets (salt and pepper appearance)
<i>E. maxima</i>	Jejunum, Ileum	++	Distended intestine (ballooning) with hemorrhagic spots, abundance of yellow-orange mucoid discharge
<i>E. brunetti</i>	Cecum and Rectum	+++	Thickening of the mucosa, coagulative necrosis with a caseous eroded surface over the entire mucosa. Coagulated blood and mucosal casts are apparent in the droppings
<i>E. tenella</i>	Cecum	+++	Presence of prominent blood and severe hemorrhage with white red spots. Cecal cores, which are accumulations of clotted blood, tissue debris, and oocysts in birds surviving the acute stage

Table 2. Lesion Scoring for highly pathogenic *Eimeria* of chicken.

<i>Eimeria</i> species	Lesion score	Macroscopic lesions
<i>E. acervulina</i>	1	Scattered, white plaque-like lesions containing developing oocysts. These lesions are elongated with the longer axis transversely oriented on the intestinal walls like the rungs of a ladder. They may be seen from either the serosal or the mucosal intestinal surfaces. They may range up to a maximum of 5 lesions per cm ² .
	2	Lesions are much closer together, but not coalescent; Lesions may extend as far posterior as 20 cm below the duodenum. The intestinal walls show no thickening. Digestive tract contents are normal.
	3	Lesions are numerous enough to cause coalescence with reduction in lesion size and give the intestine a coated appearance. The intestinal wall is thickened and the contents are watery. Lesions may extend as far posterior as the yolk sac diverticulum.
	4	The mucosal wall is greyish with colonies completely coalescent. Congestion may be confined to small petechiae or, in extremely heavy infections, the entire mucosa may be bright red in color. Individual lesions may be indistinguishable in the upper intestine. Typical ladder-like lesions appear in the middle part of the intestine. The intestinal wall is very much thickened, and the intestine is filled with a creamy exudate, which may bear large numbers of oocysts. Birds dying of coccidiosis are scored as 4.
<i>E. maxima</i>	1	Small red petechiae may appear on the serosal side of the mid-intestine. There is no ballooning or thickening of the intestine, though small amounts of orange mucus may be present.
	2	Serosal surface may be speckled with numerous red petechiae; the intestine may be filled with orange mucus; little or no ballooning of the intestine may be present; and a thickening of the wall may be observed.
	3	Intestinal wall is ballooned and thickened. The mucosal surface is roughened; intestinal contents are filled with pinpoint blood clots and mucus.
	4	The intestinal wall may be ballooned for most of its length; it contains numerous blood clots and digested red blood cells are giving a characteristic color and putrid odor; the wall is greatly thickened. Dead animals are scored as 4.
<i>E. tenella</i>	1	Very few scattered petechiae on the caecal wall are observed; there is no thickening of the caecal walls; and normal caecal contents are present.
	2	Lesions are more numerous with noticeable blood in the caecal contents; cecal wall is somewhat thickened; normal caecal contents present.
	3	Large amounts of blood or caecal cores are present; caecal walls greatly thickened; little, if any, faecal contents in the caeca.
	4	Caecal wall is greatly distended with blood or large caseous cores; fecal debris are lacking or included in cores. Dead birds are scored as 4.

The prevention and control of coccidiosis is based on the use of prophylactic anticoccidial drugs, vaccines, natural feed additives, and strict biosecurity on farms.

Control with anticoccidial agents

In spite of the challenges posed by drug resistance anticoccidial drugs still remain the primary means of coccidiosis control worldwide. Anticoccidials may be classified into two categories according to their origin synthetic compounds, which are produced by chemical synthesis and have a specific mode of action against the metabolism of the parasite and polyether or ionophore antibiotics, which are produced by the fermentation of *Streptomyces* spp. or *Actinomadura* spp., which generally destroy coccidia by interfering with the passage of monovalent or divalent ions including sodium, potassium, calcium and magnesium, through the cell membrane of parasites (Witcombe *et al.*, 2014). The polyether ionophores became the drugs of choice for prevention of coccidiosis in 1972 and remain the most extensively used today. In market there are “mixed

products” that are comprised of a combination of the two. The choice of product depends upon the type of birds, season of the year, type of program or other factors which affect exposure. In broilers the objective is to produce the maximum growth and feed efficiency with minimum of disease. In long-lived birds like table-egg layers and breeders kept on the floor, the objective is to protect against early acute infections and to provide long-lasting immunity. List of commonly used anticoccidials is given in table 3 (Mesa-Pineda *et al.*, 2021)

Fermentation-derived ionophore and chemically synthesized anticoccidials remain the backbone of coccidiosis control. Nevertheless, like the majority of antimicrobials, their repeated use in poultry production over time and the lack of new active ingredients in recent years has caused an increase in the resistance of strains of *Eimeria* spp. (Bafundo *et al.*, 2008; Peek and Landman, 2011). These resistances bring with them an increased risk of subclinical coccidiosis appearance, an increase in intestinal dysbiosis and bacterial enteritis

Table 3. Commonly used anticoccidials in chicken.

	Category	Anticoccidial agent	Recommended dose (ppm)-Broiler
Chemicals	Monovalent	Monensin	100-120
		Narasin	60-80
		Salinomycin	44-66
	Monocyclic glycosidic	Maduramicin	5-6
		Semduramicin	25
	Divalent	Lasalocid	75-125
		Amprolium	125-250
		Aprinocid	60
		Clopidol	125
		Decoquate	30
		Diclazuril	1
		Dinitolmide (zoalene)	125
		Halofuginone	3
		Nequate (methyl benzoquate)	20
		Nicarbazin	125
		Robenidine	33
Mixed	Synthetic with ionophore	Salinomycin/nicarbazin	50
		Narasin/nicarbazin	54-90 (combination)
		Maduramicin/nicarbazin	3.75-40
		Semduramicin/nicarbazin	15-40
		Monensin/nicarbazin	40
	Synthetic with synthetic	Meticlorpindol/methylbenzoquate	110

such as Necrotic Enteritis and a consequent worsening of productive parameters. To overcome this, anticoccidials are now used under programs called dual (or shuttle) or straight rotation. In the first program, two or more anticoccidials, usually with different modes of action, are alternated in the different feeds supplied during the chicken life cycle; whereas in the second program, the same drug is used continuously throughout a production cycle, but is changed for an alternative drug after one or several flocks (Quiroz-Castaneda *et al.*, 2015).

Control of coccidiosis in chicken by vaccination

The primary strategy for preventing and controlling avian coccidiosis has become the vaccination with either live or attenuated vaccines following the implementation of antibiotic bans throughout the world (Williams 2005). The efficacy of the vaccine is highly dependent upon the species of *Eimeria* contained within the vaccine (Shirley *et al.*, 2005). These vaccines normally contain 3 or more species of *Eimeria*, which are most important. In the case of broiler vaccines, these are *E. acervulina*, *E. maxima*, and *E. tenella*. Due to the geographical diversity of *Eimeria* isolates, vaccine efficacy can differ significantly (Smith *et al.*, 2002).

A number of new vaccines have emerged recently, including recombinant vaccines, DNA vaccines and genetically engineered live vaccines. The first vaccines

against coccidia used live, wild-type, sporulated *E. tenella* oocysts and were initially marketed in the 1950's (Peek and Landman, 2011). Live vaccines use the oral introduction of low doses of *Eimeria* oocysts to stimulate humoral and cellular responses from the host immune system. The ingested oocysts generate a variety of antigens in different stages of the *Eimeria* life cycle to stimulate the cellular response being the most important in terms of resistance to the disease. Virulent strains isolated from the field without any alteration in their pathogenicity, being highly effective and relatively cheap to produce. However, this practice also risks compromising flock performance and occurrence of clinical disease if managed incorrectly (Blake *et al.*, 2021). There are several virulent live oocyst vaccines for avian coccidiosis including CocciVac®-B, CocciVac®-D, VACM®, ADVENT®, Inovocox®, Inovocox®-EM1 developed in the United States (Cai *et al.*, 2022). Immucox C1 and Immucox C2 developed in Canada, as well as Nobilis COX-ATM developed in New Zealand (Peek, 2010).

Attenuated vaccine is used extensively in Europe as well as parts of Africa, Asia and Australasia, primarily with layer and breeder stock owing to their relative cost and limited productive capacity (Blake *et al.*, 2020). Paracox-5 and Paracox-8 are the first two early-

maturing live avian coccidiosis vaccines which were developed in 1989 (McDonald and Shirley, 2009). Since then Livacox D®, Livacox T®, *Eimeriavax* 4M, Inmuner Gel-Coc, Hipracox Broilers and other early-maturing live attenuated vaccines have been developed and marketed successively (Shahid *et al.*, 2020).

To address the emergence of drug-resistant strains of *Eimeria*, recombinant vaccines have been considered as an alternative strategy for controlling coccidiosis. CoxAbic was the first commercially available coccidiosis subunit vaccine which contains affinity purified antigens (APGA) isolated from *E. maxima* that are capable of controlling coccidiosis in the sexual stage (gametocyte). Since then many potential vaccine antigens have been identified and reported viz. α -Tubulin (Ding *et al.*, 2008), profilin (also called 3-1E) Lee *et al.*, 2010), microneme 1 (MIC-1) (Qi *et al.*, 2013), immune mapped protein 1 (IMP1) (Yin *et al.*, 2015), elongating factor 1 α (EF-1 α) (Lin *et al.*, 2017), microneme 3 (MIC-3) (Huang *et al.*, 2018), apical membrane antigen 1 (AMA1), gametocyte 22 (Gam22) (Rafiqi *et al.*, 2019), Rhomboid-Like Protein 3 (ROM3) (Chen *et al.*, 2022), rhoptry kinase family protein 17 (ROP17) (Liu *et al.*, 2022). These recombinant proteins can stimulate the chicken body to produce partial immunity to one or more *Eimeria* spp. However, as the recombinant antigen is unable to trigger a wide range of protective immune responses like the live or attenuated parasite vaccine, there are some limitations in using a single recombinant *Eimeria* protein to prevent coccidiosis.

DNA vaccine

Several antigenic proteins have been evaluated in animals in the form of DNA vaccines, and relevant research results have shown that DNA vaccines can significantly reduce the shedding of fecal oocysts (Ding *et al.*, 2008). At present, there are several types of coccidiosis DNA vaccines have been studied, including 3-1E, SAG1 (also known as TA4 antigen, lactate dehydrogenase (LDH, MIC2, MIC7, Rhomboid protein 2 (ROM2), 14-3-3, surface antigen 16 (SAG16) and SAG22, SAG6 and SAG15. It is gratifying that DNA vaccines are less expensive compared to the recombinant vaccines. However, DNA vaccines may integrate into the host chromosome and even induce a long-lasting strong cellular immune response, affecting host cell growth (Zaheer *et al.*, 2022).

Natural products for Coccidiosis Control

Public pressure to reduce drugs in the food chain has led to search for alternative strategy to the control of coccidiosis. Among the alternative measures explored is the use of natural products which include probiotics, prebiotics, essential oils and organic acids.

Probiotics

Probiotics are usually used as live microbial feed supplements, which are ingested by chickens which can

protect the integrity of the intestinal epithelial barrier and reduce oocyst shedding after being infected by *Eimeria* spp. Lactobacillus-based probiotics, Primalac found effective in reducing the oocyst shedding and increased T and B cell specific cytokine against *E. acervulina* infection (Dalloul *et al.*, 2005). The effectiveness of *Pediococcus* based probiotics in improving performance against *E. acervulina* and *E. tenella* infection is also reported (Lee *et al.* 2007). Since then, a consistent report on the use of probiotics like *Saccharomyces cerevisiae* (Gao *et al.*, 2009), *Bifidobacterium* and *Enterococcus faecium* (Giannenas *et al.*, 2012), *B. subtilis* (Wang *et al.*, 2019), *Pediococcus pentosaceus* (Yulianto *et al.*, 2021), *Clostridium butyricum* (Cai *et al.*, 2022) either alone or together with other supplements has been confirmed to reduce microscopic lesion in birds infected with coccidiosis (Chen *et al.*, 2016). Mohsin *et al.*, (2022) reported higher cell mediated immune response and lower oocyst per gram count in *Lactobacillus plantarum* fed broiler chicken infected with *Eimeria tenella*. Dietary P8 supplementation found effective in reducing oocyst shedding and improved the growth performance in the early infection of coccidia (Wang *et al.*, 2021).

In addition, the combined application of probiotics and avian coccidiosis vaccine can also help to improve the protective effect of the vaccine and enhance the production performance parameters of broilers (Mohsin *et al.*, 2022). Therefore, probiotics have significant application prospects as adjuvants for avian coccidiosis vaccines. Supplementation of BLES, a probiotics product with coccidiosis vaccine represents a promising strategy for improving growth performance, alleviating clinical manifestations and inducing favorable alterations to the intestinal microbiota in broiler chickens affected by coccidiosis (Cai *et al.*, 2023).

Prebiotics

Prebiotics are metabolites produced by probiotics that selectively stimulate the growth of beneficial bacteria in the gut system and increase the number of beneficial microbiotas. Clostat HC. SP Dry®, Arabinoxylooligosaccharides (AOS), fructooligosaccharides (FOS), isomaltooligosaccharides (IMOS), mannan-oligosaccharides (MOS), soy oligosaccharides (SOS), xylooligosaccharides (XOS), pyrodextrins, and inulin are used in poultry farming (Garba *et al.*, 2022; Lee *et al.*, 2022). These prebiotics shed oocyst in feces and reduces the severity of lesions in chicken orally infected with three *Eimeria* mixtures (*E. acervulina*, *E. maxima* and *E. tenella*) (Elmusharaf *et al.*, 2007). Therefore, the use of prebiotics is emerging as a new approach to control coccidiosis.

Plant-derived compounds

Plant-derived adjuvants mainly include essential oils and organic acids. Plant-derived compounds, like essential oils (EO) and their bioactive derivatives, have

recently gained popularity as antimicrobial and anti-inflammatory compounds, and have increasingly been used to help ensure the intestinal health of animals. The use of EO has shown an inhibition toward the various stages of *Eimeria* life cycle (Bozkurt et al., 2016). Some of the common EO like oregano, carvacrol, thymol, and cinnamaldehyde have shown good antiprotozoal activity against *Eimeria* (Bozkurt et al., 2012; Muthamilselvan et al., 2016; Attree et al., 2021; Felici et al., 2021). The essential oils of *Origanum vulgare* and *Citrus* spp. have been extensively studied to control co-infections of *E. maxima*, *E. acervulina*, *E. tenella* in poultry (Dudko et al., 2018). A mixture of cashew nut liquid and castor oil improved weight gain and feed conversion in broilers challenging with *E. maxima*, *E. acervulina* and *E. tenella* (Moraes et al., 2019). Use of 2% coconut oil in broiler feed improved growth performance and villus histology during coccidial challenge (Hafeez et al., 2020). Puvaca et al., (2020) reported effectiveness of dietary tea tree essential oil in increasing hen-day egg production and reducing coccidia oocysts output. Curcumin extracted from *Curcuma longa*, may be useful to reduce the lesions caused by *E. acervulina* and *E. maxima* in upper and middle part of small intestine.

Combination of carvacrol, cinnamaldehyde, and capsicum oleoresin are helpful in stimulating cell-mediated immunity resulting in an increase in natural killer cells, macrophages, CD4, CD8 T cells, and cytokines such as IFN- γ and IL-6 that increased the host immunity against coccidiosis by stimulating the innate as well as the adaptive (humoral) immune response (Naidoo et al., 2008). An extract from *Artemisia annua*, Artemisinin have shown promising results in the prophylaxis of coccidiosis (Coroian et al., 2022). Recently (Felici et al., 2023) reported effectiveness of EO deriving from basil (BEO), garlic (GAR), oregano (OEO), thyme (TEO), and their main bioactive compounds *in vitro* against the invasion of *Eimeria tenella* sporozoites and in modulating primary immune response.

Organic acids which mainly include short-chain fatty acids (SCFAs), Glycerol monolaurate (GML), Butyric acid glycerides (BAG, Lactobutylin) and clopidol, etc. Acetic acid has shown effective protection in chickens infected with *Eimeria* spp. (Balta et al., 2021; Khan et al., 2022).

CONCLUSIONS

For the control and prevention of poultry coccidiosis proper hygienic and bio security measures should be implemented, and prophylactic anticoccidial drugs should be provided with appropriate time and recommended dose. Information gained from molecular assays can guide the poultry farmers in managing disease by allowing informed decisions on which anticoccidial compounds (traditional or naturals) or live oocyst

vaccines should be used in the field. Phytochemical compounds can be used as preventive, therapeutic, or immuno-modulators against coccidiosis.

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Gut microbiome, gut health and chicken performance and options to modulate gut microbiome

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ABSTRACT

The chicken gut microbiota is important for host immune system development, competitive exclusion of pathogens and host nutrition. Highly competitive poultry production system necessitates maintaining healthy gut to obtain best performance in poultry. Broiler chickens are mostly reared under deep litter system wherein reuse of litter as bedding material and improper handling of litter poses high risk of infection from carryover pathogens. Farmers often use low cost in feed antibiotics in sub-therapeutic doses to ward off infection risk and often as a safeguard against substandard bio security arrangements and also to improve feed efficiency. Prolonged uses of low dose of antimicrobial growth promoters (AGP) are of huge societal concern due to their potential to increase antimicrobial resistance (AMR), and increases risk to human and animal health. Various attempts have been made to identify microbial diversity in different breeds and identify microbes associated with performance and modulate early colonization of gut with desired microbial consortium. Various alternatives to antimicrobial growth promoters have been shown to support comparative or better performance than that of AGPs, including additional benefits like stimulation of immunity or suppression of coccidia, fungi or viruses, but they are generally costlier than AGPs. Multipronged approaches such as developing a reliable certification system and higher pricing of poultry products produced through use of alternatives to antibiotic growth promoters, improving availability of veterinary services including making culture and sensitivity tests mandatory before prescription of antibiotics, improving quality of water and vaccines, *etc.* offer huge opportunity to modulate gut microbiome favourably without use of antibiotics.

Keywords: Chicken, gut, gut health, microbiome

INTRODUCTION

Achieving good health, better feed efficiency and a faster growth rate is crucial for a highly competitive poultry production system. For attaining these goals, maintaining a healthy gut is an important prerequisite. The gastrointestinal tract of chickens is densely populated with complex microbial communities (bacteria, fungi, archaea, protozoa, and viruses) with bacteria being the predominant one. The interactions between the host and the chicken gut microbiome are considered to play important roles in bird nutrition, physiology, and gut development. An empirical study proved the importance of commensal gut microbes in the well-being of host animals, wherein it was shown that a germ-free mouse can be killed with 10 cells of *Salmonella enteritidis*, although it requires 10⁶ cells to kill a conventional mouse (Collins and Carter, 1978). The intestinal tissue has a very high turnover rate to allow it to expand in size and function or nutrient uptake capacity. Hence, about 19.3 to 50% of oxygen utilized in the whole body is utilized in the intestine (Cant *et al.*, 1996). Further, the intestine is one of the most active organs of the immune system housing more than 70% of resident immune cells of the entire body (Vighi *et al.*, 2008). Poor gut health in chickens has been associated with gut wall morphology changes, inflammation, systemic infection, and malabsorption. The importance of gut health in chicken production can be gauged from the fact that more than 50% of the therapeutic use of antibiotics in broilers in the European Union, is to control gut pathogens (Persoons *et al.*, 2012). About 40 percent of globally produced antibiotics are used as growth promoters (Hughes and Heritage, 2004). It has been suggested that the amount of antimicrobials used in food animals is expected to escalate globally from 99,502 tonnes in 2020 to 107,472 tonnes by 2030 (Mulchandani *et al.*, 2023). In intensive

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broiler farming antimicrobials are often administered in sub-therapeutic doses for prolonged periods (36 days or more) as growth promoters (FAO, 2018). These conditions favor the selection and spread of resistant bacteria within animals and to humans through food or other environmental pathways (Benani *et al.*, 2020; Sohail *et al.*, 2021). In a recent study, it has been shown that feeding antibiotic growth promoters for consecutive three cycles resulted in a significantly increased abundance of the antibiotic resistance genes (ARG) in the gut microbes of chickens on a farm including the density of

some of the ARGs of structurally unrelated antimicrobials (Paul *et al.*, 2022). Once the administration of antibiotics ceases, the loss of resistance is a slow process. Even after the antibiotic is removed from the environment, some bacteria populations can retain their antibiotic resistance for extended periods (Luo *et al.*, 2005). Antimicrobial resistance (AMR) is an issue of global health as well as economic importance. The bacteria resistant to antimicrobials in animals that are of the highest potential risk to human health are likely to be zoonotic pathogens transmitted through food, like *Salmonella*, *Campylobacter*, etc. In addition, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), extended-spectrum beta-lactamase-producing *E. coli* (ESBL *E. coli*), and mobile colistin-resistance gene (*mcr-1*) have shared antibiotic resistance genes (ARGs) at the human-animal interface. It is estimated that bacterial AMR was directly responsible for 1.27 million global deaths in 2019 (ARC, 2022).

Most of the broiler birds in India and other tropical countries are reared under a deep litter system where carryover of pathogens in bedding material from one production cycle to another adds to pathogen load in the gut and contributes to a higher risk of occurrence of diseases. Unrestricted use of various broad-spectrum antibiotics as a therapeutic agent is also common in case of viral infections or any illness in poultry production systems without the establishment of a causative agent. Recent research carried out by Paul *et al.*, (2022) indicated that many ARGs are ubiquitous in the gut microbiome of broiler chickens and AGPs favor selection of ARGs structurally related as well as unrelated classes of the AGP. Hence, identification of cost effective alternative to antibiotic growth promoters is in focus. In this review, we will focus on the diversity of the gut microbiome of chicken, the role of the gut microbiome in the gut health and performance of chicken, and the current state of knowledge on dietary supplements to modulate gut health favorably and reduce the use of antimicrobial growth promoters or therapeutic use of antimicrobials.

Colonization and succession of chicken gut microbiome and its modulation

The gut is colonized by microorganisms around hatching. One study showed that the initial colonization of the gut of birds began with the passing of microorganisms from the dam through the pores of the eggshell (Lee *et al.*, 2019). Immediately after hatching, lots of environmental microbes try to get established within the gut but the host plays a very important role in the selection of its symbionts in the gut through immune response and other attributes like pH, passage rate, etc. In one study, the first bacteria detected to colonize the caeca within the first four hours of life were streptococci and enterobacteria, which were then found to be distributed

throughout the intestinal tract within 24 hours, and on day three, many more bacteria such as lactobacilli, enterococci, and coliforms could be isolated from the different parts of the gut (Coloe *et al.*, 1984). Already inhabited microbes also play an important role in the selection of other microbes (Baumgartner *et al.*, 2020). Also, some microbes have been shown to have evolved mechanisms for evading host immune response (de Jong and Alto, 2018) and have developed complex metabolic profiles to make them fit into particular environments to make them compatible with other microbes within the ecosystem. A succession of microorganisms in the gut is observed in which the species richness and diversity of the community structure of the microbiota increase as the birds grow until the microbiota reaches a state of stabilization. In the case of commercial broiler chickens, this stabilization period is around three weeks of life (Diaz-Carrasco *et al.*, 2019). However, stabilization times and succession patterns of gut microbiota can vary greatly depending on the genetic makeup of the birds, farm management factors, environment, and nature of feed (feed processing, feed components, feed buffering activity, and feed additives), (Choi *et al.*, 2014). Proteobacteria were found at higher density during the first few days of life and thereafter decreased. However, in older birds, Firmicutes were the most dominant phyla present (Gong *et al.*, 2002). It has been suggested that early colonization of the intestine is of great importance for poultry health and productivity (Kers *et al.*, 2018). The inoculations with prebiotics, probiotics, and consortia of microorganisms applied directly in ovo have been shown to support the early intestinal colonization of birds with a "healthy" microbiota immediately after hatching (Simon *et al.*, 2016; Baldwin *et al.*, 2018). However, the in-ovo technique has not found its place in practical use due to its invasive and cumbersome nature. Recently it was shown that feeding a compound probiotic comprising *Bifidobacterium*, *Lactobacillus*, *Streptococcus faecalis*, and yeast) for the first 24 d in broiler chickens increased abundance of beneficial bacteria *Barnesiella*, reduced abundance of harmful bacteria and drug resistance genes as compared to antibiotic control (Feng *et al.*, 2023). The reuse of litter (a mixture of bedding material and bird excreta) during consecutive flocks is a common practice that is usually applied to reduce costs in broiler production and alleviate the challenge of litter disposal. Generally, poultry litter can be reused for a year or longer if managed well and maintained in a relatively dry state but there is concern that the reused litter environment may negatively affect the gut microbiota of birds as chicks start pecking and ingesting litter materials from the first day of life. It has been shown that fresh litter contains more environmental bacteria, while reused litter contains more bacteria of intestinal origin (Cressman *et al.*, 2010). The success of poultry litter reuse in the US

can be attributed to scientific treatment methods used by farmers during the time lapse between consecutive cycles such as decking, tilling, treatment with acid-based amendments, etc to reduce ammonia and bacterial load. In one study, it has been shown that reused litter does not lead to higher abundances of *C. perfringens* or *Salmonella* (Wei *et al.*, 2013). However, chickens raised on fresh litter showed increasing colonization with beneficial microbes such as *Lactobacillus* spp., while a group of bacteria yet to be classified within Clostridiales dominated in the ileal mucosal microbiota in the reused-litter chicks. (Cressman *et al.*, 2010). Wang *et al.* (2016) reported that the reuse of litter increased the prevalence of halotolerant/alkaliphilic bacteria in the litter, but also altered the intestinal microbiota of the birds, increasing the caecal levels of *Faecalibacterium prausnitzii*, a butyrate-producing species. Bucher *et al.* (2020) showed that the physicochemical properties of litter such as ammonia, PH, moisture, water activity, and aluminum, besides microbiome can exert an influence on pathogen abundance in the litter. They also showed that a consortium of *Bacillus subtilis* strains isolated from the litter reduced the growth of *Salmonella enterica* serovar Heidelberg (SH) in vitro. Most of the studies on the relation between the gut microbiome and litter reuse

are from developed countries, which may not reflect the Indian situation as in India most of the small farmers may not follow scientific treatment of litter. Detailed studies are needed to assess how litter reuse can influence chicken gut health, especially on Indian farms.

Microbial diversity in the gut of chicken

The caecum and hindgut of chickens harbor a complex microbiome. Early cultivation-based studies indicated a high density (about 10^{10} to 10^{11} /g microscope counts) of anaerobic bacteria in the caecum of chicken (Salanitro *et al.*, 1974). In a study involving bacterial census of chicken gut microbiome based on 16S rRNA gene sequences submitted in GenBank, Pan, and Yu (2014) reported that more than 900 species-equivalent operational taxonomic units (OTUs) representing 117 genera were found in chicken. The most predominant genera found were *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides*. In another study, the microbial community composition of 8 varieties of local aboriginal Jiangxi breeds of chicken was analyzed by Li *et al.*, (2023) in China using 16S rRNA gene amplicon sequencing and they reported 1340 OTUs with an average number of 769 OTUs per sample. In this study, the chicken gut microflora was mainly

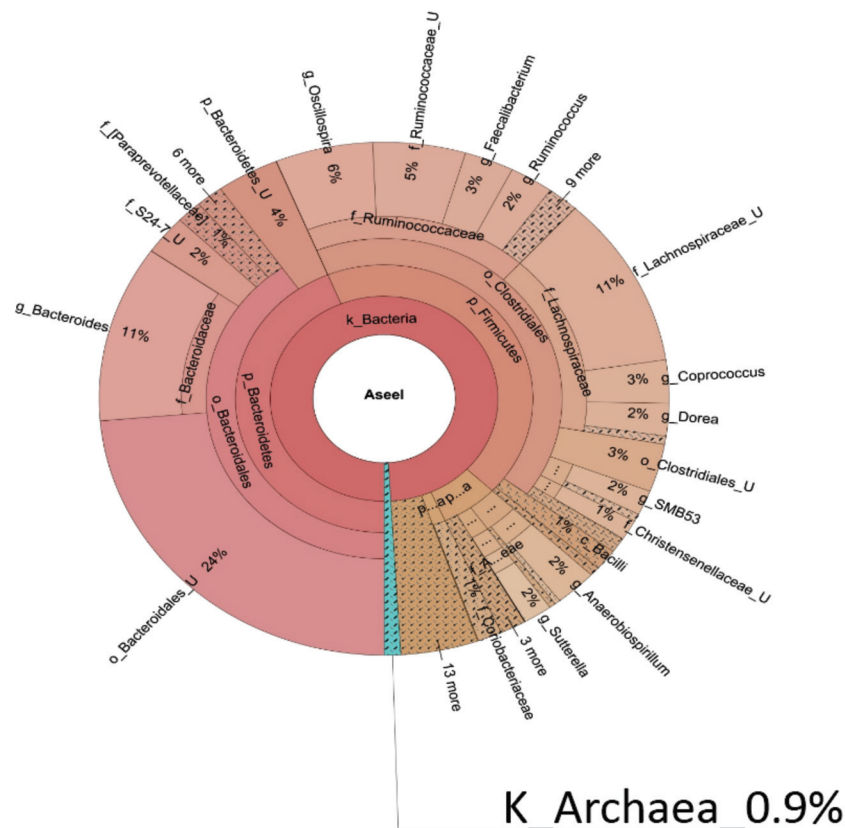


Fig 1. Krona chart of taxonomic assignments of 16S amplicon sequencing reads of metagenomic DNA of gut content of Aseel breed of chicken indicating mean relative abundance of the prokaryotes at different taxonomic levels (Paul *et al.*, 2021)

composed of Firmicutes (68.51%), Bacteroides (11.63%), Proteobacteria (9.46%), and Actinomyces (3.45%) and 8 genera with relative abundance of more than 2% were identified as *Lactobacillus* (31.2%), *Romboutsia* (9.22%), *Clostridium_sensu_stricto_1* (4.6%), *Enterococcus* (3.47%), *Gallibacterium* (2.43%), *Bacteroides* (2.37%), *Turibacter* (2.35%), and *Rikenellaceae_RC9* (2.09%). In an earlier study conducted by us using amplicon sequencing of 16S rRNA genes of the gut microbiome of Indian chickens, the indigenous Aseel breed had the highest number of non-singleton nondoubleton OTUs (3461), followed by Ghagus (1911), and Nicobari (1767) among native breeds, whereas, commercial broiler Vencon430 had 857 OTUs (Paul *et al.*, 2021). In Aseel, the gut microbiota was dominated by Bacteroidetes (44%) followed by Firmicutes (43%), Proteobacteria (6%), Actinobacteria (1%), and Cyanobacteria (0.8%) constituted 94.8% of the whole phyla (Fig 1). In the broiler line Firmicutes, Bacteroidetes, Cyanobacteria, Proteobacteria, and Verrucomicrobia were the major phyla, which accounted for 81, 6, 6, 4, and 1% of total sequences, respectively. The study also indicated that *E.coli* was the most abundant bacteria and *Lactobacillus* was one of the most abundant beneficial bacteria with a known probiotic role in broiler chickens.

Relation between gut microbial composition and health and performance of hosts

The gut microbiome is considered the second genome of the host (Grice and Segre., 2012), and is linked to a range of functions such as in immune stimulation and programming of host immune system development, competitive exclusion of pathogens or non-indigenous microbes, and host nutrition (Aruwa *et al.*, 2021). Oakley *et al* (2014) showed that gut microbes stimulate the development of cytotoxic and helper T cells, immunoglobulin-producing cells and phagocytic cells, and the lamina propria, which prevent invasion of opportunistic pathogens. The gut microbiota's attachment to the epithelial walls of the intestine reduces the opportunity for the colonization of pathogenic bacteria (Yegani and Korver, 2008). In the hindgut, the microbiota also produces short-chain fatty acids (SCFA), vitamins (e.g., vitamin K and vitamin B groups), and amino acids from the undigested feeds, which eventually become available for the host. SCFA produced in hindgut supplies approximately 20- 30% of the energy requirement for maintenance in nonruminants (R  rat *et al.* 1987, Bergman, 1990, Yen *et al.*, 1991). SCFA can also kill or suppress pathogens, and can further stimulate gut epithelial cell proliferation, thus increasing the area of absorption surface of villi. In addition, gut microbiota can convert uric acid to ammonia, which is subsequently absorbed by the bird and further used to produce amino acids such as glutamine (Singer, 2003) and influence bile

salt metabolism and recycling (Suzuki, 1970).

However, the gut microbiome can also be a reservoir of bacterial pathogens including those having potential zoonotic roles such as *Salmonella* and *Campylobacter* which can harbor antibiotic resistance genes and mobile genetic elements. In the gut, microbes may produce toxic metabolites, and degrade bile acids, which in turn decrease the growth and fat digestibility of the birds, respectively (Gaskins *et al.*, 2002). Further, microbes in the gut increase mucin secretion, increase the rate of epithelial cell turnover and increase the secretion of IgA. These processes although beneficial for the prevention of attachment of pathogens to the intestine, do increase the demand for energy and protein from the host.

Recent use of high throughput sequencing technologies and qPCR has helped to understand gut microbiome composition about feed efficiency. Based on such analysis, *Lactobacillus salivarius*, *Lactobacillus aviarius*, *Lactobacillus crispatus*, *Clostridium lactatifermentans*, different members of family Ruminococcaceae, *Bacteroides vulgatus*, *Akkermansia*, and *Faecalibacterium*, among others were identified as potential performance-related bacterial species (Yan *et al.*, 2017; Torok *et al.*, 2011; Han *et al.*, 2016). In another study, eight families (Fusobacteriaceae, Flavobacteriaceae, Rhizobiaceae, Vibrionaceae, Xanthomonadaceae, Comamonadaceae, Campylobacteraceae, and Incertae Sedis XIII) linked to high feed conversion ratio (FCR) birds and six families (Victivallaceae, Synergistaceae, Prevotellaceae, Rikenellaceae, Enterobacteriaceae and Ruminococcaceae) associated with low FCR birds were identified (Singh *et al.* 2012). The abundances of *Escherichia/Shigella* were found to correlate negatively with growth and fat digestibility in broiler chickens (Rubio *et al.*, 2015). Wen *et al.* (2021) reported that lower abundances of duodenal *Akkermansia muciniphila* and cecal *Parabacteroides* and higher abundances of cecal *Lactobacillus*, *Corynebacterium*, *Coproacillus*, and *Slackia* were related to better feed efficiency in chicken.

Means of changing gut microbiota composition favourably

Antibiotic growth promoters (AGPs) have been shown to have a positive effect on gut microbiome and feed efficiency in most of the studies. In a meta-analysis on AGP (the most frequent antibiotics in the database were Avilamycin, Flavomycin, Virginiamycin, and Bacitracin) involving 174 scientific articles containing 183 experiments on broiler chickens, Maria *et al.* (2019) reported that higher weight gain (3.84%) and lower feed conversion ratio (3.48%) were observed in AGP fed groups during the overall experimental period (d 1 to d 42). It has been shown that many alternative feed additives can support similar or better improvement in the performance of broiler chicken, besides additional

beneficial effects on gut microbiome or immunity. Keeping in view increased societal concern about the contribution of AGPs to the development of antimicrobial resistance, many countries have banned their use and are promoting the use of alternatives.

Enzymes

Use of enzymes such as xylanase and β -glucanase are known to reduce gut viscosity and stimulate the growth of lactic acid bacteria have the ability to adhere to the gut epithelium and compete with pathogens for its colonization, besides improving digestibility, weight gain, and feed efficiency (Rodríguez *et al.*, 2012). Among non-starch polysaccharide degrading enzymes, xylanase is the most effective and commonly used. Xylanases increased the apparent ileal digestibility of amino acids by 15% in a corn-based diet and for wheat- and rye-based diets by 16% and 30%, respectively (Cowieson and Bedford, 2009). However, several studies have reported no effect of enzymes on the performance of birds (Olgun *et al.* 2018, Walters *et al.* 2018) probably due to variations in the structure of nonstarch polysaccharides and that of the ideal substrate required for the enzymes.

Phytase is commonly supplemented in poultry diets to increase the utilization of dietary phosphorus. Besides improvement in phosphorus utilization, phytase, when used at higher doses (superdosing: 2000 FTU/kg vs normal dose of 500 FTU/kg), has been shown to improve feed efficiency significantly (Carrie and Rama Rao, 2018). Superdosing degrades lower esters like IP₃, IP₄ (which are antinutrients as they bind protein, mineral, pepsin, fats, starch, etc) and supports maintaining effective dose up to lower GIT, prevents rebinding of P and inositol and facilitates inositol absorption. About 30-35% of the superdosing benefit has been suggested to be through the production of myo-inositol which plays a vital role in fat metabolism and cell function. It has been shown that phytase supplementation increases the abundance of *Lactobacillus* sp., *Clostridium leptum*, and *Enterococcus* sp. in the ileum (Ptak *et al.*, 2015). The authors stated that these findings might be associated with the greater availability of P in the lumen of the gastrointestinal tract.

Organic acids

Organic acids (OA) have been reported to exert antimicrobial activities that are similar to antibiotics and which induce significant benefits in poultry. Besides antibacterial action, OA causes a reduction in digesta pH, increases pancreatic secretion, has trophic effects on the GI mucosa, and changes the morphology of the intestinal wall in a way that reduces colonization of pathogens, thus preventing damage to the epithelial cells (Langhout, 2000; Dibner and Buttin, 2002). Sodium butyrate has been shown to favor the establishment of lactic acid-producing bacteria including *Lactobacillus* spp. and *Bifidobacterium* spp., while reducing the colonization by harmful bacteria

such as *Salmonella* and *Campylobacter* (Van Deun *et al.*, 2008). The effectiveness of different OA against different pathogens depends on pKa and the chain length of the OA. A combination of different OAs can also expand the spectrum of activity against pathogens. Fernández-Rubio *et al.* (2009) reported that sodium butyrate (in both partially protected and unprotected forms) was able to prevent *Salmonella* colonization in the crop and caeca of broilers, whereas only the partially protected source of the butyrate salt reduced colonization of internal organ (liver). Gheisari *et al.* (2007) showed that supplementation of 0.2% encapsulated OA to the diet might improve the proliferation of *Lactobacillus* spp. and reduce the population of *Clostridium perfringens*, *E. coli*, and *Salmonella* spp. in poultry gut contents.

Essential oils

Antimicrobial activities of plant extracts and essential oils (EO) have been demonstrated in several animal experiments. A mixture of EO-containing carvacrol and thymol reduced the shedding of *E. coli* and *C. perfringens* in broilers (Jang *et al.*, 2007). Siragusa *et al.* (2008) reported that adding extracts of *Humulus lupulus* into drinking water decreased the number of *C. perfringens* in the broiler intestine.

Essential oils are shown to be more effective against Gram-positive bacteria than Gram-negative bacteria. Among Gram-negative bacteria, *E. coli* is inhibited by low doses of EOs whereas *Salmonella* inhibition requires high doses of most of the EOs. Several recent studies show that mugwort, fennel, or oregano EO, as well as their components, including eugenol, isopulegol, carvacrol, carvone, and thymol, can be used in the prevention of coccidiosis in poultry (Drăgan *et al.*, 2014; Murakami *et al.*, 2014). Essential oil compounds and their combinations also show antifungal activity (Owanagh *et al.*, 2010). Saderi and Abbasi (2011) reported that thyme oil has activity against adenoviruses. Oregano oil and one of its components, carvacrol, have been demonstrated to be effective against entero- and rotaviruses (Sánchez *et al.*, 2015).

In a review (Adaszyńska-Skwirzyńska and Szczerbińska, 2017) of data from 11 feeding trials on broiler chickens involving different EOs (dose rate 75 to 1200 mg/kg feed) the increase in ADG ranged from -4 to 20.6% and improvement in FCR ranged from -23.5 to +13.2%.

In a few other studies (Basmacioğlu-Malayoğlu *et al.*, 2016; Pham *et al.*, 2022), it was shown that the use of the combination of OA and EO was more effective, in some respects than their individual use. Recently, in a study in our lab, we have shown that an immobilized blend of EO can inhibit *E. coli* while sparing beneficial bacteria in the gut thus resulting in significant improvement in feed

efficiency in broiler chicken (Paul *et al.*, 2023).

Prebiotics

Prebiotics are oligosaccharides such as fructo-oligosaccharides (FOS), galacto -oligosaccharides (GOS), mannan-oligosaccharides (MOS), and related carbohydrate polymers that can be utilized by beneficial bacteria such as *Bifidobacterium* and *Lactobacilli* but not by common pathogens. Prebiotics have 2 major modes of action. Initially, the prebiotic remain undigested in the upper part of the GI tract and are selectively utilized by some of the beneficial bacteria in the intestine and hindgut. Secondly, prebiotic fermentation results in the generation of short-chain fatty acids and lactic acid and a decreased rate of pathogen colonization (Ricke *et al.*, 2020). In the gut, xylo-oligosaccharides (XOS) were shown to increase the abundance of *Lactobacillus* and *Clostridium* cluster XIVa which is known to possess genes related to butyrate production (De-Maesschalck *et al.*, 2015). Beta-D-glucan and MOS, components of yeast cell walls have been shown to bind to the receptor mannose-specific type-1 fimbriae and prevent pathogen colonization while favoring the genus *Faecalibacterium* which is considered as beneficial bacteria for gut health (Park *et al.*, 2016).

Probiotics

Probiotics are living microorganisms that improve gut health and animal performance by competing with pathogenic bacteria for adhesion sites at the intestinal epithelium. Mechanisms of action from probiotics include enhancement of activity of digestive enzymes like proteases, lipases, and amylases, the improvement of mucosa ultrastructure, thus also increasing nutrient absorption, dislodgement of pathogens, etc (Yang *et al.*, 2017, Borda-Molina *et al.*, 2018). It has been shown that dietary supplementation with *Lactobacillus acidophilus* not only improved growth performance, but also reduced the mortality rate of broilers caused by *Escherichia coli* (Wu *et al.* 2021). It has been shown that some strains of *Bacillus* spp. can inhibit a wide range of gram-negative and gram-positive bacteria through producing antimicrobial substances (Soni *et al.*, 2021)

Synbiotics

Synbiotics denote the combined effects of pre- and probiotics. Synbiotics have been shown to improve the implantation and survival of the supplemented bacteria in the GIT (Awad *et al.*, 2009). Synbiotics were shown to be effective in the reduction of *C. jejuni*, which causes zoonosis frequently and causes a strong inflammatory effect (Humphrey *et al.*, 2014). The combination of *Bifidobacterium longum* PCB133 with an XOS successfully reduced the load of *Campylobacter* spp. and *C. jejuni* (Baffoni *et al.*, 2017). Humam *et al.* (2019) reported that supplementation with postbiotics improved growth performance, intestinal morphology,

and immune response in broiler chickens under heat stress.

Parabiotics-postbiotics

Parabiotics are defined as the inactivated microbial cells of probiotics with intact or ruptured cell components or cell extracts. Postbiotics are the complex mixture of metabolic products secreted by probiotics in cell-free supernatants such as enzymes, secreted proteins, vitamins, amino acids, peptides, organic acids, etc. These have several advantages over probiotics such as ease of production and storage, more likely to trigger only targeted response, less chance of developing and spreading antibiotic resistance or virulence gene transfer, no issue of maintaining viability, no chance to cause opportunistic infections, etc (Nataraj *et al.*, 2020). Kareem *et al.* (2016) showed that the addition of postbiotics and inulin had beneficial effects on BW, feed efficiency, and mucosa architecture of broiler chickens. Similarly, supplementation of postbiotics and parabiotics in the diets of broiler chicken increased the density of beneficial microbes like Firmicutes while decreasing harmful microbes like Proteobacteria (Danladi *et al.*, 2022).

Bacteriophages

Bacteriophages are a group of viruses whose replication is dependent on the invasion of bacterial cells. Bacteriophages exhibit strong bactericidal activity. Some phages display specific affinity against particular Bacterial genera, while others have a broad spectrum of activity. The high specificity of bacteriophages may allow them only to target a specific bacterial pathogen in the gut. However, a cocktail of phages has been used to broaden their antibacterial activity. Many studies have shown the effectiveness of phages against *Salmonella*, *Campylobacter*, etc (Chinivasagam *et al.*, 2020, Li *et al.* 2020). However, problems with resistance, safety, and stability remain to be addressed for their wide application in the poultry industry (Abd-El Wahab *et al.*, 2023).

Seaweed extracts

Various plant bioactive compounds can stimulate growth and immunity in chickens and may also influence gut microbial community structure. The inclusion of red seaweed extract rich in carragenans and plant phenolics, antioxidants, and phycobilins was recently shown to increase the feed efficiency of broiler chickens significantly along with intensification of gene expression of gut immunity and porosity-related genes (Paul *et al.*, 2021).

Improving water and feed quality

Water quality is considered important for proper digestion and maintaining a healthy gut environment. The pH of water should be maintained slightly acidic, between 5.5 and 7, as the optimum activity of most digestive enzymes is achieved in this range. When the

pH value of the crop is above 6.5 the enzyme activity can be reduced 10–15% compared to the effectiveness observed at pH 4.5–5.5 (Kierończyk *et al.*, 2016). High concentrations of salts and total solids in the water and basic pH generally favor the production of biofilm and endotoxins in water lines. Poor water quality may increase the use of antibiotics and thus promote antibiotic resistance. Dietary calcium levels higher than the requirement can be deleterious to intestinal health since it may chelate nutrients, reduce enzyme activity, and promote the proliferation of *Clostridium* bacteria (Paiva *et al.*, 2013).

Dietary fat for poultry should be free from rancidity. The diet should be free from mycotoxins. Quality control for soybean meal is also important. Under-heated soybeans have higher levels of antitrypsin factor whereas overheating decreases protein digestibility.

Improving biosecurity and hygiene and development of effective vaccines

There is an urgent need to train farmers on biosecurity measures. Also, there is an urgent need to develop more effective vaccines against viral and bacterial diseases, which will go a long way to reduce the use of AGPs. Notably, in one study on 64 farms in 9 European countries, the majority of pig operations experienced cost reductions for antibiotic treatments after *L. intracellularis* vaccination (Adam, 2009). In the case of poultry, it has been suggested that the development of new or improved vaccines for *E. coli*, infectious bursal disease virus, *Clostridium perfringens* type A, *Eimeria*, and Infectious bronchitis virus may reduce antibiotic use dramatically (Hoelzer *et al.*, 2018)

CONCLUSIONS

The gut microbiome plays a vital role in the health and performance of broiler chickens. There is a wide difference in the gut microbiota of native and broiler chickens. Antibiotic growth promoters as well as therapeutic use of antibiotics have been shown to facilitate the development of antimicrobial resistance in the gut microbiome of chicken with the potential exchange of such resistance to other microbes affecting the health of animals and humans through environmental interface. Recent studies have indicated the widespread presence of antimicrobial resistance in Indian poultry farm environment. Numerous alternatives to AGPs have shown promising and equivalent or better results than AGPs in experimental trials although they are costlier than AGPs. Bans on growth promoters within Europe led to little or no impact on livestock productivity and disease, including in poultry, yet changes in the management of farms such as improvements in hygiene and biosecurity, and associated costs are not available in the public domain. Behind such success in

Europe, extensive stewardship, improving veterinary service, reliable certification system and higher pricing of products contributed to convincing farmers to use alternatives to AGPs instead of AGP. These results are difficult to extrapolate to current antimicrobial use and the potential impacts of change in antimicrobial use on AMR and livestock feed systems in India. Extensive efforts towards developing a reliable certification system and higher pricing of poultry products produced through alternate systems, improving availability of veterinary services including making culture and sensitivity tests mandatory before prescription of antibiotics, and increasing consumer awareness can be a way forward.

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Chicken infectious anemia: A silent threat to poultry health

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ABSTRACT

Intensive rearing systems for poultry, especially chickens, face a persistent threat from Chicken Infectious Anemia (CIA), a virus that emerged in the 1970s, rapidly gaining global prevalence. This review highlights the significant impact of CIA on poultry health and productivity. Originating from contaminated vaccines in Japan in 1979, Chicken Anaemia Agent (CAA) evolved into Chicken Anemia Virus (CAV). Contrary to assumptions, CAV's presence in the United States dates back to 1959, challenging the perception of recent emergence. CIAV, belonging to the Gyrovirus in the Circoviridae family, infects chickens globally, particularly in regions with intensive poultry farming. Transmission occurs vertically and horizontally, affecting chicks before two weeks of age. Beyond causing anemia and immunosuppression, CIAV exacerbates co-infections, leading to increased susceptibility to various pathogens. Late seroconversion in Specific Pathogen-Free (SPF) flocks poses challenges for vaccine manufacturing. Pathogenesis involves causing severe tissue and organ damage, impacting adaptive immunity and inducing immunosuppression through cytokine modulation. Clinical signs encompass ruffled feathers, lethargy, gangrenous dermatitis, and mucous membrane discoloration, with significant mortality within 5-6 days. Postmortem findings reveal macroscopic lesions such as pale bone marrow and subcutaneous hemorrhages. Histologically, severe lymphoid depletion occurs in primary and secondary lymphoid organs. Diagnosis involves history, clinical symptoms, postmortem findings, and laboratory tests such as ELISA. The control of Chicken Infectious Anemia Virus (CAV) is crucial due to its potential to diminish the immune response against various vaccine viruses, including NDV, MDV, ILTV, and FPV. Failure to control CAV can result in vaccination failures or worsen the residual pathogenicity of attenuated vaccine viruses. Effective control measures are essential, emphasizing strict sanitary practices, immunization, and the utilization of various vaccines to prevent and mitigate the impact of CAV on poultry health and production.

Keywords: Chicken Infectious Anemia, immunosuppression, lymphoid depletion, secondary infection

INTRODUCTION

Poultry population, especially chicken, has been exposed to intensive rearing system over the years to meet the ever-increasing demand for their meat and eggs. But this system experiences constant threat from various types of potentially harmful viruses which evolve in frequent intervals. Highly intense systems of poultry rearing pave way for emergence and re-emergence of different viruses globally during the last few decades. Chicken Infectious Anemia (CIA) is one such poultry virus infection emerged in 1970's and attained high global prevalence within few years.

The infection is caused by Chicken infectious anemia Virus (CIAV) which is a small, non-enveloped, icosahedral virus belonging to the genus Gyrovirus of the family Circoviridae containing a single stranded circular DNA genome (Pringle *et al.*, 1999). Chicken are the natural hosts for CIA and the infection severely affects chicken of initial 3 weeks by targeting the lymphoid organs and hampers the immune system. This will lead to immune-suppression and chicks will succumb to secondary complications and associated mortality. Since the lymphoid organs will be damaged by the virus, chicks which survive the infection in early weeks will remain immune-compromised, making them vulnerable to infections and reduction in productivity. Hence the presence of CIA may not be evident in poultry flocks but it has the potential for reduced productivity, frequent infections and vaccine failures. Considering these facts and

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the prevalence of CIA in Indian poultry, the present review has been designed to throw light on Chicken Infectious Anemia which remains as a silent threat to our poultry wealth.

HISTORY

Chicken Anaemia Agent (CAA) was initially isolated from contaminated vaccines in Japan following inoculation in chicken; and later its associated condition, chicken infectious anemia, was documented in 1979 (Yuasa *et al.*, 1979). Subsequently, isolation of CAA could be done from the UK

(Chettle *et al.*, 1989; De Wit *et al.*, 2004), Australia (Firth and Imai, 1990), and Sweden (Engstrom *et al.*, 1988). CAA was later proposed to be renamed Chicken anemia Virus (CAV) (Gelderblom *et al.*, 1989). The presence of this virus infection in the United States was established as early as 1959 through the analysis of archived sera from chickens utilized for alloantisera production (Toro *et al.*, 2006). These serological results support assumptions, based on circumstantial evidence, that CAV must have been present in the United States long before its first isolation. The collective findings from these studies indicate that CAV is not a recent virus, transmitted through Marek's disease (MD) vaccination but rather an "ancient" virus that has existed for an extended period without causing overt issues (Goryo *et al.*, 1985).

EPIDEMIOLOGY

The virus is prevalent globally and particularly common in regions with intensive poultry farming. It's important to note that chicken infectious anemia is not a recent disease but a newly acknowledged one (Pope, 1991), occurring both in commercial flock and Specific Pathogen Free (SPF) flocks. Transmission occurs through vertical and horizontal routes (Hoop, 1992), (Yuasa and Yoshida, 1983). Horizontal transmission occurs *via* contact with chicks infected vertically, as well as through contaminated fomites, housing, and similar sources. Infection with Chicken Infectious Anemia Virus (CIAV) in chickens infected before 2 weeks of age, lacking maternal antibodies to CIAV, can lead to severe anemia, bone marrow aplasia, lymphoid depletion, subcutaneous hemorrhages, immunosuppression and increased susceptibility to multiple secondary bacterial infections (von Bülow and Schat, 1997). While it was believed that infection in birds older than 3 weeks had minimal consequences, CIAV infection has been causally linked to Marek's disease (MD) outbreaks (Miller and Schat, 2004). CIAV induces suppression in immune system, leading the chicks more susceptible to secondary infections. In older birds CIAV infection has been associated with the generation of cytotoxic T lymphocytes (CTL) to other pathogens, suggesting a pivotal role in the development of additional diseases (Markowski-Grimsrud and Schat, 2003). Co-infection of Infectious bursal disease (IBD) (Cloud *et al.*, 1992), Marek's disease (Otaki *et al.*, 1987), leads to profound immunosuppression, increasing susceptibility to a broader range of viral and bacterial pathogens. Another significant consequence of CIAV infection is the challenge of preventing infection in both commercial and noncommercial specific-pathogen-free (SPF) flocks. Seroconversion in SPF flocks often occurs around or after the onset of reproductive maturity, resulting in substantial losses to the SPF industry and vaccine manufacturers. The reasons behind late

seroconversions in SPF flocks remain poorly understood. Due to the potential vertical transmission of Chicken Anemia Virus (CAV), it is crucial for Specific Pathogen-Free (SPF) flocks, responsible for providing eggs for vaccine manufacturing, to remain free from CAV infection. This is necessary to prevent the contamination of avian vaccines with CAV (Todd *et al.*, 1999, Miller and Schat, 2004). Enzyme-linked immunosorbent assay (ELISA) is the preferred test for examining the epidemiology of Chicken Infectious Anemia Virus (CIAV) infection in poultry due to its speed and suitability for testing a large volume of serum samples (Todd *et al.*, 1999).

PATHOGENESIS

Chicken infectious anemia virus (CIAV) can spread through both vertical and horizontal transmission. Vertical transmission occurs when the virus is passed from breeders to their offspring, while horizontal transmission involves the spread through feathers, oral contamination, and feces (Miller and Schat, 2004). The virus primarily affects young chickens lacking maternal antibodies in the first two weeks of life, causing significant tissue and organ damage. Chicks with maternally inherited antibodies are protected from vertical transmission, but they can still experience horizontal transmission with subclinical symptoms as the antibody levels decline (Davidson *et al.*, 2008). This results in stunted growth and more prone to various secondary infections such as Infectious bursa disease, Marek's disease, and other adenoviral infection (Natesan *et al.*, 2006). The severe consequences of this virus encompass lymphoid atrophy, reduced body weight, bone marrow aplasia, muscular depletion of red blood cells (RBC), and the onset of anemia (Yuasa *et al.*, 1980).

CAV replication primarily occurs in hemocytoblasts located in the bone marrow, precursor T cells situated in the thymus cortex, and dividing CD4 and CD8 T cells within the spleen. The initial cytolytic infection in Hemocytoblasts and lymphoblasts occurring at six to



Fig 1. Chicks of 1 week age infected with CIAV.

eight days post-infection, resulting in the swift depletion of these cells through apoptosis. In addition to enlarged proerythroblasts and deteriorating hematopoietic cells, the bone marrow has been noted to contain macrophages that have engulfed degenerated hematopoietic cells.

T cells are important target of CIAV, which has an impact on the adaptive immunity. Although B cells are not directly susceptible to CAV, damage to cytokines and other molecules has been linked to the virus's indirect effects on B cells. (Adair, 2000). The primary mechanism of CAV is immunosuppressive activity leading to decrease of cytokines like interleukin 2 (IL-2), which has an indirect impact on neutrophils, macrophages, and the phagocytic activities. (Natesan *et al*, 2006; Oluwayelu *et al*, 2010). Interferon gamma (IFN- γ) has been shown to rise in the early stages of infection and then gradually decline in addition to suppressing immunological molecules. (Natesan *et al*, 2006). According to the several researches, after 15 days of infection, the blood, thymus, and spleen had the highest viral load, while the liver, bone marrow, and bursa showed the least amount of viral expression. Significant decreases in cytokines (IL-2, IL-1, and IL-12) at all dosages were also observed, along with an initial 3–15-fold rise in IFN- γ during the early stages of infection. There have also been reports of CAV-infected chicks having lower levels of CD4+ and CD8+ (Adair, 2000; Wani *et al*, 2016). It is also possibilities that viral may shed on other organs and cause infection on lung, proventriculus, kidney and ascending portion of the duodenum. Generally, it takes more than 22 days following infection at one day of age to detect infected cells in these tissues (Smyth *et al*, 1993).

CLINICAL SIGNS

The clinical signs of the disease first appear when the infected birds are 7 to 14 days of age (Todd, 2000). The disease is clinically characterized by ruffled feathers, lethargy and gangrenous dermatitis of the skin. Mucous membranes are pale and there is presence of cutaneous hemorrhages, subcutaneous haemorrhages and intramuscular haemorrhages (Adedeji *et al*, 2016, Ganar *et al.*, 2017). After the onset of acute form there is peak mortality within 5 to 6 days of disease. The mortality declines after 5 to 6 days (Engstrom and Luthman, 1984). In Infected Birds the mortality rate is upto 55% and morbidity of the disease is up to 80% (AboElkhair *et al.*, 2014). Anaemia is a characteristic with marked thrombocytopaenia and decreased hematocrit values in birds which are 2 to 4 weeks of age (Kamdi *et al.*, 2016).

The lesions can also be found in the Bursa, thymus spleen and Bone marrow (McNulty, 1991), however, in other visceral organs the lesions can also be found (Todd *et al.*, 2000). These lesions may be mostly related with the secondary infections (Miller and Schat, 2004).

In CIA infected birds the most common findings are pale and fatty bone marrow of femur and atrophy of haematopoietic elements with severe depletion of erythropoietic and granulopoietic tissue. Lipocytes has been found to replaced haemopoietic cells in severe cases (Dhama, 2002). There have been reports of severe to moderate lymphoid depletion in spleen of CIA infected birds, as well as localised apoptosis and necrosis. Bursa revealed moderate necrosis of bursal follicles, proliferation of reticular cells and lymphoid depletion (Kamdi *et al.*, 2016). In many CIA outbreaks nephrosis in kidneys, swollen and mottled spleen and liver, pale and icteric carcass been reported (Balamurugan *et al.*, 2006, Gowthaman, 2014).

POSTMORTEM FINDINGS

The Macroscopic lesions, more commonly observe at necropsy are a pale or yellowish bone marrow which is due to anaemia, haemorrhages in proventriculus mucosa or subcutaneous haemorrhages, severe atrophy of the thymus. The subcutaneous haemorrhages on the wing may be complicated by secondary infection and gangrene causing blue appearance (Amin and Amer, 2021). Thymic atrophy, often causing a near-complete absence of thymic lobes, is the most common lesion, especially in chicks that develop age resistance to anemia (Jeurissen, *et al*, 1992, Schat and van Santen, 2020). Fabricius of bursa might be smaller in size. Haemorrhages can occur under the skin. Secondary infections may also cause lesions.

Microscopic lesions

Histologically, there is severe atrophy and depletion of lymphoid cells in the spleen, bone marrow, thymus and bursa of Fabricius. In addition to this, intranuclear inclusion bodies of CAV are also found in haemocytoblasts of the bone marrow and macrophages of thymus (Sonkusale *et al*, 2022).

Significant depletion of lymphoid cells in both primary and the secondary lymphoid organs. Severe depletion of lymphocytic cells is seen in thymic cortex. Additionally, the erythrocytic and granulocytic part of bone marrow also shows atrophy or hypoplasia. Adipose tissue replaces haematopoietic cells and Hyperplasia of bone marrow occurs between 24 and 32 days after infection in recovered birds. There is severe lymphoid depletion in the thymus. Both the cortex and medulla of thymus become equally atrophic, followed by hydropic degeneration and occasional necrotic foci present in thymus. In the lymphoid follicle of spleen there is depletion of T cells with hyperplasia of reticular cells. Sometimes liver cells are swollen, and there may be dilation of hepatic sinusoids (Schat and vanSanten, 2020).

DIAGNOSIS

Chicken infectious anemia can be tentatively diagnosed by the history, clinical symptoms, and the postmortem findings while, the accurate diagnosis is possible either via isolation and identification of virus, demonstration of virus antigen in impression smears or tissue sections (Dhama *et al.*, 2008) and also by the demonstration of CIAV specific antibodies in the serum. Isolation of CIAV can be done either in cell culture or in embryonated egg, but the process is very cumbersome, required skilled personals and is costly too. lymphoblastoid cell line originated from the Marek's disease tumour such as, MDCC-147 and MDCC-MSB1 are suitable for the isolation of CIAV (Schat *et al.*, 2008). The cytopathic effect shown by the virus infected cell culture includes high number of dead cells, enlarged swollen cells, change in pH of media to alkaline, and the difficulty to proceed the serial subculture after 1-6 sometimes up to 10 passage (McNulty, 1991). Other than virus isolation, molecular techniques including PCR, q-PCR and in-situ hybridization can also be employed for detecting the presence of viral DNA. PCR is a frequently used molecular technique for detecting the presence of viral DNA in the tissue sample of birds and is considered as more sensitive and specific when comparing with isolation of virus in cell culture and other methods (Miller *et al.*, 2005). The presence of virus antigen in various tissues can be demonstrated by immunohistochemistry or immunofluorescence technique. Serum antibody can be detected by techniques such as virus neutralization test (VNT) or by ELISA (Shettima *et al.*, 2017).

CONTROL MEASURES

Chicken infectious anemia virus (CIAV) is often considered as a hardy virus to control once it is

introduced into the poultry flock, due to its resistant nature to many of the common disinfectants. Moreover, it is very difficult to diagnosis the infection in adult birds as it is subclinical in nature. So, the best solution to prevent the disease is not letting the virus in to the farm premises. It can be achieved by the implementation of strict sanitary measures and immunization regimens. With the use of highly effective vaccines against CIAV, the disease can be controlled to a certain extent. Generally, clinical form of CIAV infection is common only in chicks, and they can be protected by maternal antibodies which are transferred from the immunized breeder parent and it last for a period of up to three weeks (Todd, 2000). Usually it is recommended that, breeder flock should be immunised with commercially available live attenuated vaccine at an age in between 9 and 15 weeks (Schat *et al.*, 2008). Vaccine can be either administered as injection or via drinking water. Once the maternal antibodies wane, again chicks are exposed to infection, but this time in a subclinical form which will complicate the prevention and control of CIAV. Also, it should be taken into consideration that, co infection with other immunosuppressive infection such as IBD, MD can worsen the immune status of the bird, and simultaneously increases the pathogenicity of CIAV virus (Owoade *et al.*, 2010), thereby control of such disease should be an integral part of CIAV control program.

Different vaccine approach have been attempted for chicken infectious anemia virus including live attenuated vaccine, inactivated vaccine, subunit vaccine and DNA based vaccine

Inactivated vaccine

Inactivated vaccines are favored over live attenuated vaccine, considering the risk of later being revert to virulent through recombination events with other virulent strains (Sawant *et al.*, 2015). As CAV is prevalent

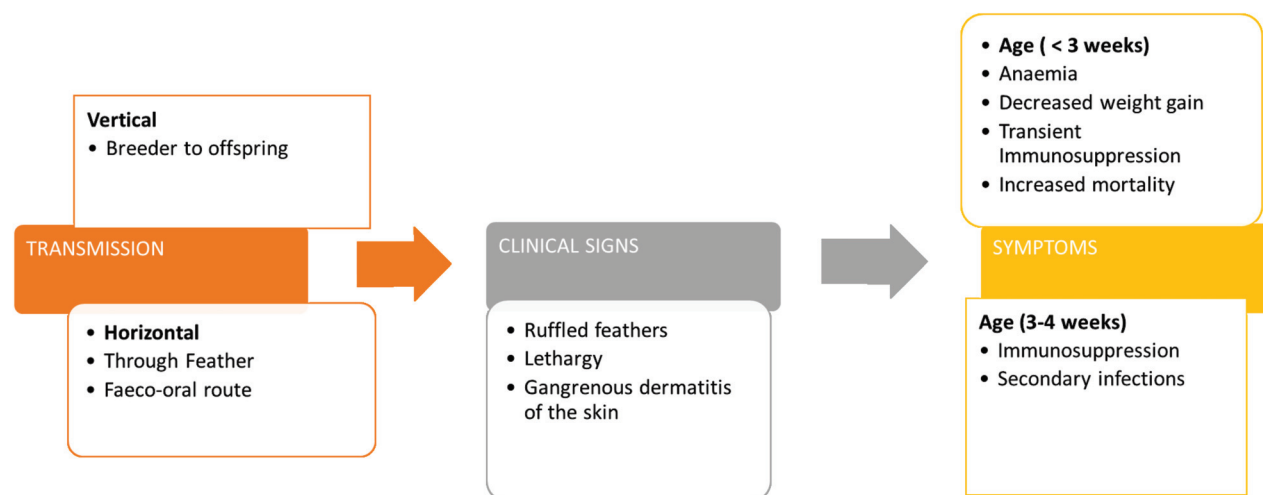


Fig 2. Schematic representation of CIA: Transmission and clinical signs.

globally, the possibility of emergence of new virulent virus strain via the use of live vaccine is also high. Inactivated vaccines are safer to use against the attack of virulent CIAV (Zhang *et al.*, 2015), but are not able to generate a strong immune response. In addition, low titre of CIAV in chicken embryo and other cell line, high production cost associated with inactivated vaccine etc. make it as a non-feasible strategy for commercialization throughout the world. In 2015, Zhang *et al* pioneered to develop an inactivated vaccine candidate from a highly virulent CIAV isolate, GD-G-12 through propiolactone hydrolysis and found that high antibody titre could be developed among vaccinated chickens and their chicks and suggested it as a potential vaccine candidate.

Subunit vaccines

In recent years, vaccine research on CIAV has been increasingly focused towards the development of genetically engineered vaccine such as subunit vaccine. Currently different expression system including both eukaryotic and prokaryotic expression system has been used to express various proteins such as VP1 alone or VP1-VP2 protein (Noteborn *et al.*, 1998; Lacorte *et al.*, 2007). Several research findings shown that, humoral immune response generated by the VP1 protein alone is low, whereas the co expression of VP1 and VP2 could generate a strong antibody response (Koch *et al.*, 1995; Noteborn *et al.*, 1998). Moreover, adjuvants such as oil emulsion adjuvant and CpG adjuvant are frequently incorporated to enhance the immune response induced by subunit vaccine (Fang *et al.*, 2019).

DNA based vaccine

As an alternative to live attenuated and inactivated vaccine, DNA vaccine can be considered for immunizing the chicken against CIAV. Here, instead of using the whole virus, a small portion of the viral genome which encodes a viral protein that contain the neutralizing epitope is selected. There are several advantages associated with DNA vaccines, such as the ability to induce both cell mediated and humoral immune response, ability to generate immune response even in the presence of maternal antibody, and absence of residual pathogenicity. However, there are certain limitation exist such as vaccine cannot spread far away from the initial inoculation site thereby leading to a low immune response. To counteract the drawback, several strategies has been tried to improve the suboptimal immune response associated with DNA vaccine. In 2015, Sawant *et al* used HMGB1 C protein as an adjuvant to enhance the immune response and it was observed that a higher immune response could be elicited in the vaccinated group in which HMGB1 C protein was used as adjuvant.

Every category of CIA vaccine has its own benefits and drawbacks, and it will be beneficial to use various types of vaccines based on the prevailing situations. Live

attenuated type of vaccine cannot be employed in a place where chicken infectious anaemia is endemic as it can lead to the emergence of new virulent strain. Absence of high viral titre and high production cost limit the use of inactivated vaccine. DNA vaccine is not able to induce strong antibody response whereas subunit vaccines are inefficient to stimulate CMI response. Therefore, it would be wiser to establish a standard regimen that incorporates both DNA vaccine and subunit vaccine thereby ensuring a complete immune response.

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An insight into important bacterial and fungal diseases in ducks

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ABSTRACT

Duck rearing has been practiced in many parts of the country, especially in the wetland ecosystems of south-central Kerala, Assam, West Bengal, Odisha, Andhra Pradesh, Karnataka and Tamil Nadu. This is practiced either in a traditional manner or as a backyard system and supports to a certain extent the poultry industry. Many species of local ducks are also valued as ornamental birds. Duck farming for generations remained as the source of livelihood for a sizeable population. Duck farming has definitely contributed a lot to the local economy in the form of products, employment generation and is considered as an important source of high-quality animal protein for the local community. Although it is a lucrative poultry industry, certain years have been marred by disease outbreaks of various natures which seriously culminated in disaster. This could be bacterial, viral, fungal, or even parasitic infections with mortality ranging from 10% to 60 %. The most important bacteria affecting ducks are *E. coli*, *Pasteurella*, *Riemerella*, *Salmonella*, *Streptococcus* and *Staphylococcus*. Among the fungal pathogens, *Aspergillus fumigatus* still has a major toll in duck industry. This review systematically analyses and summarizes the pathology of important bacterial and fungal diseases of ducks prevalent in India.

Keywords: Duck, Bacteria, Fungus, Pathology, Diagnosis

INTRODUCTION

Duck farming has a crucial role in the agricultural economy of Asian continent which alone accounts for 82.6% of the entire duck meat production of world (Jha and Chakrabarti, 2017). This lucrative sub-poultry industry contributes through its egg, meat and feather. The duck meat and eggs form a major component of high-quality protein in the diet among the rural population in the water-logged areas of many developing Asian countries. Duck eggs are relatively larger, weighing about 4.5% of duck's body weight compared to chicken, whose egg weight is 3.3% of the hen's body weight (Narahari, 2009). In India, duck farming is an important tool for alleviating poverty among the rural communities and has great potentials in tribal areas (Jha and Chakrabarti, 2017). Ducks being more prolific and better adaptable to free-range system of rearing with simple needs of housing is preferred to chicken among the rural population (Rajput, *et al.*, 2014). There are over 10 million ducks in India, which form only about 10% of the total poultry population in the country and contribute about 6-7% of the total eggs produced in the country. In India, ducks are mostly reared in states like West Bengal and Assam in the eastern part and Tamil Nadu, parts of Andhra Pradesh, coastal Karnataka and Kerala in southern region. The availability of vast riverbeds and enormous water bodies are contributing in the development of duck industry. Every state in the country has its own local breed of duck, which have been evolved naturally over decades with high disease resistance. More than forty breeds of ducks have been reported in India which are native to many states. Arni, Chara, Chemballi, Gadwall, Khazana, Mithilli, Nagi, Pati *etc.* are a few varieties of desi breeds of ducks in India (Morduzzaman *et al.*, 2015; Reena *et al.*, 2019). As a result of inbreeding, many of the dominant characters of these breeds, including the disease resistance were compromised. This review tries to analyze the important bacterial and fungal diseases of ducks in the country.

BACTERIAL AND FUNGAL DISEASES IN DUCKS

Sporadically, bacterial infections have resulted in high mortality and

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carcass condemnation which culminated in huge economic loss in the duck industry. Bacterial diseases cause higher mortality rates in ducks than viral diseases. Even though many bacteria are documented as pathogens in ducks, only few imparts major threat to this industry viz. *Escherichia coli* (*E. coli*), *Pasteurella multocida* (*P. multocida*), *Riemerella anatipestifer* (*R. anatipestifer*), *Salmonella species*, *Staphylococci* and *Streptococci*. Among fungal etiologies, *Aspergillus species* creates havoc in duck industry and effects duck of any age group, unlike chicken, where it is mainly limited to the brooding stages.

Duck colibacillosis

Colibacillosis caused by *E. coli* is reported as the most common bacterial infection of commercial ducks causing significant and largest economic loss to the poultry industry (Liu

et al., 2019). The study by (Adzitey *et al.*, 2012) indicates that ducks like other farm animals are primary reservoirs for *Escherichia coli* including potential pathogenic types. An investigation of heavy mortality in an organized duck farm in Kashmir valley revealed the presence of avian pathogenic *Escherichia coli* (APEC) (Qureshi *et al.*, 2010). *E. coli* causes a wide variety of problems in ducks at different ages, but the most dangerous illness occurs at 2-6 weeks of age in which mortality rates reach up to 43%. The age group mentioned applies when the ducks are reared under the confinement systems viz. intensive system of rearing on deep litter. Five different serogroups including O1, O18, O111, O78, and O26 were considered as the most important etiological agent in infections of ducks (Eid *et al.*, 2019) whereas O158, O103, O125, O44, O114, O91, O111 and O78 were isolated by (Roshdy *et al.*, 2012). Being an opportunistic and commensal organism, it can be a primary causal organism or secondary to other infections such as viruses or management problems which may affect the flock. Clinical signs in affected birds can vary from sudden death to birds being 'off colour' with their necks pulled into their bodies. *E. coli* O-groups were found to be associated with different diseases such as septicemia, salpingitis, peritonitis, arthritis, omphalitis and tumor-like masses in the intestine and liver (Khalid, 1990). *E. coli* was obtained from 6% of the duck carcass with arthritis, serogroup 078 being most prevalent (Bisguard, 1981). The presence of a soft cough is also suggestive of infections with *E. coli*. Postmortem signs are very similar to those in the fowl or turkeys. Acute infections may only show a congested carcass, congested lungs and small haemorrhages on the heart and air sacs. Studies revealed mortality of 11.0% due to *E. coli* in growing ducks affected with hemorrhagic enteritis and extensive epicardial hemorrhages (Islam *et al.*, 2004). *E. coli* was also isolated from cases of salpingitis (Bisguard, 1995) manifested as hemorrhagic follicular inflammatory exudations and peritonitis, leading to reduced egg production and high culling of breeder duck flocks. However, more chronic infections will show the characteristic lesions of pericarditis, perihepatitis, enlargement of liver, air sacculitis and pneumonia. In breeding birds, the presence of peritonitis and salpingitis is very common. An investigation on duck *E. coli* infection in the Mekong delta revealed that 990 from 994 ducks were confirmed to be infected by *E. coli* and the highest rate of positive isolates was reported from liver (78.3%), followed by lungs (71.8%), spleen (67.4%), and the lowest one was in bone marrow (58.9%) (Ho *et al.*, 2019). Bariha *et al.*, (2019) reported that *E. coli* (55%) could be isolated from 112 number of samples such as liver (22), lungs (30) and swab from bone marrow (38) of dead birds. Of the three respiratory tract disease syndromes that occur in birds associated with *E. coli* infection, acute colisepticemia, characterized by

hyperemic and swollen viscera, tended to occur in young birds. Subacute fibrinopurulent serositis involving air sacs and pericardium was more common in older birds. Chronic granulomatous pneumonitis was not seen as flock enormities but as chronic disease in birds dying in small numbers sometime after one of the previously mentioned forms of the disease (Cheville and Arp, 1978). Histopathological examination of trachea shows sloughing of mucosa and accumulation of cellular debris. In lungs, histopathological lesions are characterized by interstitial pneumonia and infiltration of inflammatory cell especially heterophils, mononuclear cell, increase in the thickness of alveolar septa and most of the cases, emphysema in the lung tissue and severe hemorrhage (Al-Hially and Al-Hamadany, 2019) during concurrent infection of *E. coli* and avian influenza. Liu *et al.*, (2019) reported that co-infection with APEC, H9 AIV and Tembusu virus (TMUV) is very common. Diagnosis is based on the postmortem findings and a rapid growth of the organism on blood or MacConkey or Tryptic Soy agar, gene specific multiplex PCR and sequencing (Devigasri *et al.*, 2018).

Duck Pasteurellosis

Pasteurellosis caused by *P. multocida* is a contagious and endemic disease causing high morbidity and mortality in the affected domestic and wild birds (Ganti, 2003). The disease known as Duck cholera in ducks are extremely common across the world and is also a recognized cause of duck mortality (Hunter and Wobeser, 1980; Pehlivanoglu *et al.*, 1999; Punnoose *et al.*, 2021; Amonsin *et al.*, 2002). This organism is very common in Asia and Middle East countries. It has been demonstrated that birds older than 11 weeks are less vulnerable to *P. multocida* infection than young ones (Hunter and Wobeser, 1980). Studies demonstrated the age susceptibility of ducks that 8-week-old village ducks had more severe clinical signs, followed by 4-week-old ducks. Clinically, fowl cholera could be picked between days 1 and 5 in ducks. The incubation period varied from 12 to 48 hours with 100% mortality rate between 24 to 72 hours of infection (Islam *et al.*, 2003). It is also reported that age range of affected ducks was from 2-16 weeks, susceptible age ranged from 30-100 days (Awadin *et al.*, 2017). Awadin *et al.* (2017) reported mortality rate from 33.3 to 46.6%. A survey conducted in Bangladesh on the causes of duck mortality reported that about 11 percent of total death of domestic ducks was due to duck cholera (Baki *et al.*, 1991;1993). The disease was experimentally reproduced in young and adult ducks by oral infection with culture of *P. multocida*, originally isolated from a case of duck cholera. Mortality following experimental infection varied from 50 to 100 percent depending on the dose of inoculums (Baki *et al.*, 1991).

Experimental infection of groups of ducks of 1-2

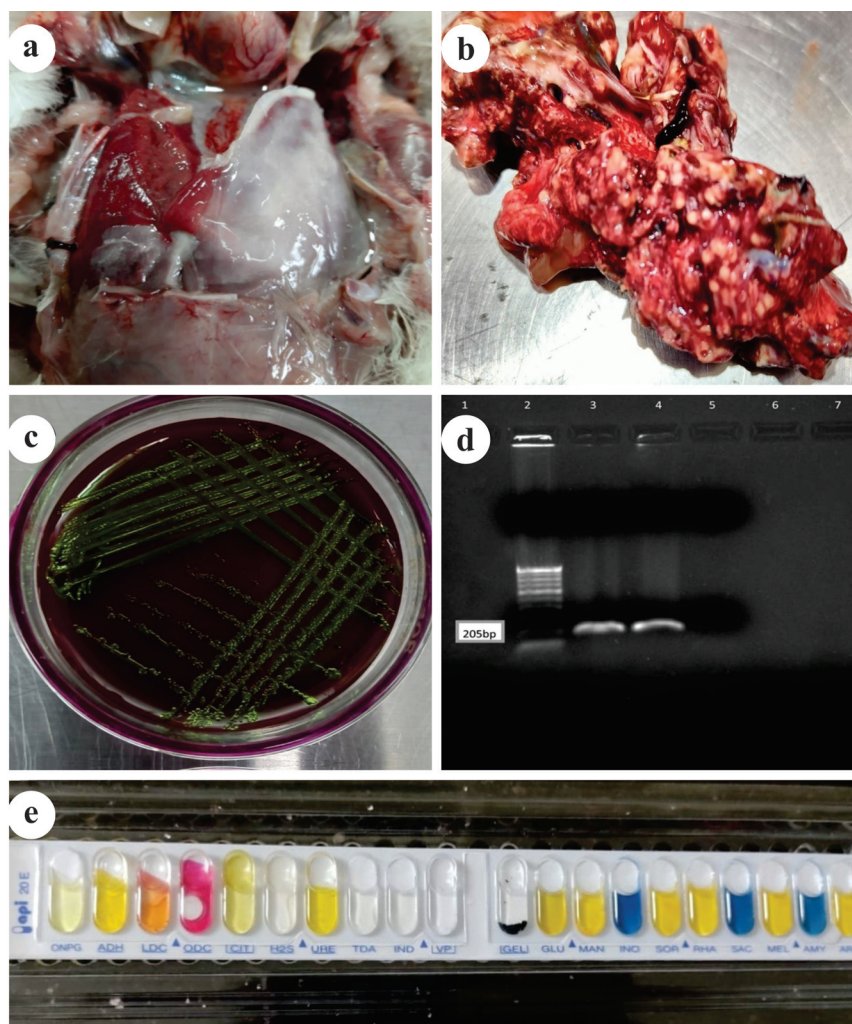


Fig 1a. Riemeerellosis - Severe perihepatitis, pericarditis and airsacculitis in a 3 week old bird. **b.** Lung showing multiple caseated nodules in aspergillosis infected duck. **c.** Isolation of *E.coli* in EMB agar showing greenish metallic sheen. **d.** PCR amplification of 16s RNA of *Riemeerella anatipestifer* showing 205 bp product. Lane 2 represents 100 bp ladder, 3 represents DNA template, 4 and 5 represents positive and negative control respectively. **e.** API 20E for *E.coli*. **e.** Antibiotic Sensitivity.

months and 5 months old at two different doses 0.25 ml and 0.5 ml reported that incubation period was 3–4 hours in young ducklings, while in 5-month-old ducks; it was 6–8 hours. Clinical signs were similar in both young and adult with huddling, ruffled feathers, coughing, oculo-nasal discharge, diarrhea and death within 10–26 hours in ducklings and 26–41 hours in older ducks (Pramod *et al.*, 2011, Prabhu *et al.*, 2019). There was progressive reduction of hemoglobin, total erythrocyte count and leukocytosis with increase numbers of heterophil (Pathak *et al.*, 2011., Pramod *et al.*, 2011). Signs in acute outbreaks can be just sudden death in large numbers of birds. In more chronic infections, signs of depression, conjunctivitis and dyspnoea can occur. Fever, anorexia, mucous discharge from the mouth and nostrils, diarrhea, ruffled feathers, decrease in body condition, lameness and unable to hold the head with beak touching the ground before death, soiling of cloaca, yellowish nasal discharge and

increased respiratory rate can be observed (Islam *et al.*, 2003). Acute duck cholera is characterized by severe watery diarrhea, anorexia, respiratory manifestations and septicemia, while chronic one leads to localization of the organism in certain organs. Affected birds were found to be in good body condition and had gross lesions of enlarged spleens and multifocal areas of hepatic necrosis. Ducks that died acutely of duck cholera had developed lesions of hemorrhagic septicemia with widespread vascular damage and focal necrosis in spleen, liver and other organs. Ducks that survived the challenge developed chronic disease with lesions in a variety of organs including brain, lungs, air sacs, joints and eyes. In some birds, bacteria gets localized and acts as a carrier of disease (Hunter and Wobeser, 1980).

At necropsy, the gross lesions are mainly observed in heart, lungs, liver, spleen and duodenum. There were many focal necrosis on the surfaces of liver, so called

corn meal liver and spleen. Lungs show congestion and edema. The duodenal mucosa appears swollen, hyperemic and hemorrhagic. Epicardial hemorrhages are common. Haemorrhage and congestion throughout the visceral organs, serofibrinous exudates in lungs and cytoplasmolysis in cardiac fibers and hepatocytes were the principal pathological changes observed in both natural experimental cases of duck cholera (Baki *et al.*, 1991). Remnants of fibrin and fibrous strands on the air sacs, pericarditis and perihepatitis, fibrosis on the lungs, necrotic spots on the spleen, liver, increased amount of peritoneal and pericardial fluids could be observed (Pathak *et al.*, 2011., Pramod *et al.*, 2011). Petechial and ecchymotic hemorrhages were common, particularly in subepicardial and subserosal locations, hemorrhages on the coronary band of heart, hemorrhages on air sac membranes adjacent to lungs can also be observed. Gross examination of intestinal lumen revealed thickened yellowish necrotic mucous membrane, hemorrhagic enteritis and ecchymotic hemorrhages at the ileocecal junction, mostly observed as annular bands. Louis *et al.* (1970) recorded unilateral ecchymotic hemorrhage at the site of esophageal-proventricular junction. *P. multocida* could be isolated from 60% of the ducks showing respiratory manifestations and watery diarrhea. Of the isolated species, 69.8% of the isolated *P. multocida* strains followed capsular type A serotype, 20.9% of capsular type D and 9.3% as untypable. Lungs and air sacs had the highest incidence of organism followed by liver, heart and spleen. *P. multocida* serogroups highly expressed virulence attributes responsible for diarrhea (Eldin and Reda, 2016). Swollen hock joint and thickened foot pad were grossly detected in ducks only (Awadin *et al.*, 2017). Trachea and lungs were severely congested and haemorrhagic and serofibrinous exudates were seen in the lung, liver and heart (Islam *et al.*, 2003). Histopathologically, the lesions were characterized by the cellular degeneration and coagulative necrosis of liver, acute splenitis, the epicardial hemorrhages and cellular degeneration in heart, hemorrhagic and catarrhal duodenitis, congestion and edema of lungs. Large numbers of colonies of bacteria and bacterial emboluses could be observed in liver, spleen and duodenum. The histopathological lesions in the infected ducks were reported to be more severe than those detected in the infected chickens that characterized by multiple granulomata in most examined organs (Awadin *et al.*, 2017). Diagnosis is relatively straightforward by bacterial culture, although the organism can be masked by overgrowth of bacteria such as *E. coli*.

Duck Riemerellosis

Riemerellosis is an enzootic, contagious, often primary septicemic disease of domesticated ducklings (Fulton and Riemer, 2010). The disease is also known

as New Duck disease or Duck Septicaemia or Infectious Serositis and is caused by *Riemerella anatipestifer*, a gram-negative, nonspore-forming, rod-shaped and non-motile bacterium. The disease is considered to be an economically important disease worldwide, resulting in high morbidity and mortality rates in ducks. Economic loss to the duck industry from this disease is due to mortality rates ranging from 5- 75%, as well as weight loss (Hamid *et al.*, 2019). It was reported and described by Riemer, for the first time in 1904 (Segers *et al.*, 1993). The organism was then classified as *Pfeifferella anatipestifer*, *Moraxella anatipestifer* and *Pasteurella anatipestifer* (Segers *et al.*, 1993). In India, the disease is reported in Kerala (Priya *et al.*, 2008, Soman *et al.*, 2014), Assam (Doley *et al.*, 2003; Shome *et al.*, 2004; Hazarika *et al.*, 2020). A mortality rate of 16 % was reported by Shome *et al.* (2004) in Meghalaya. There are 21 serotypes of *R. anatipestifer* which may aid in epidemiological analysis (Loh *et al.*, 1992; Pathanasophon *et al.*, 1995, 2002; Chang *et al.*, 2019). Various studies reported high genetic diversity and molecular differentiation among the *R. anatipestifer* field isolates (Tsai *et al.*, 2005; Yu *et al.*, 2008). The disease is commonly observed in young ducklings aged between 2 and 6 weeks. The organism is transmitted horizontally via the respiratory route and skin. Stress factors such as movement/transportation and environmental variations can trigger disease. Different serotypes show wide variations in virulence factors (Huang *et al.*, 2002) and involvement of multiple serotypes has been reported in the same farm flock (Pala and Radhakrishnan, 2014). Signs usually include abnormal gait, head shaking and lethargy. Affected ducks develop ocular and nasal discharges, mild coughing and sneezing, tremors of the head and neck, and incoordination. In typical cases, affected ducklings may lie on their back with paddling movement of legs. Necrotic dermatitis on the lower back or around the vent may also be observed. Postmortem signs in acute cases include enlargement of the liver, spleen and lung congestion. In more chronic cases, pericarditis, perihepatitis and air sacculitis with a caseous deposit can be found. Mortality ranges from 2-50%, depending on the strain involved. Infection of the central nervous system can result in fibrinous meningitis. Birds with chronic form of the disease may develop mucopurulent or caseous salpingitis leading to loss of egg production (Dougherty *et al.* 1955; Zehr and Ostendorf, 1970; Leibovitz, 1972; Kardos *et al.*, 2007, Pala *et al.*, 2013). Histopathologically, severe fibrinopurulent inflammatory cell reaction could be observed in the affected organs and its serous membranes often with presence of multiple bacterial colonies. Diagnosis by detection of *R. anatipestifer* from clinical samples by culture and identification method is considered as the gold standard test. Examination of heart blood smears and liver impression smears reveals bipolar organisms

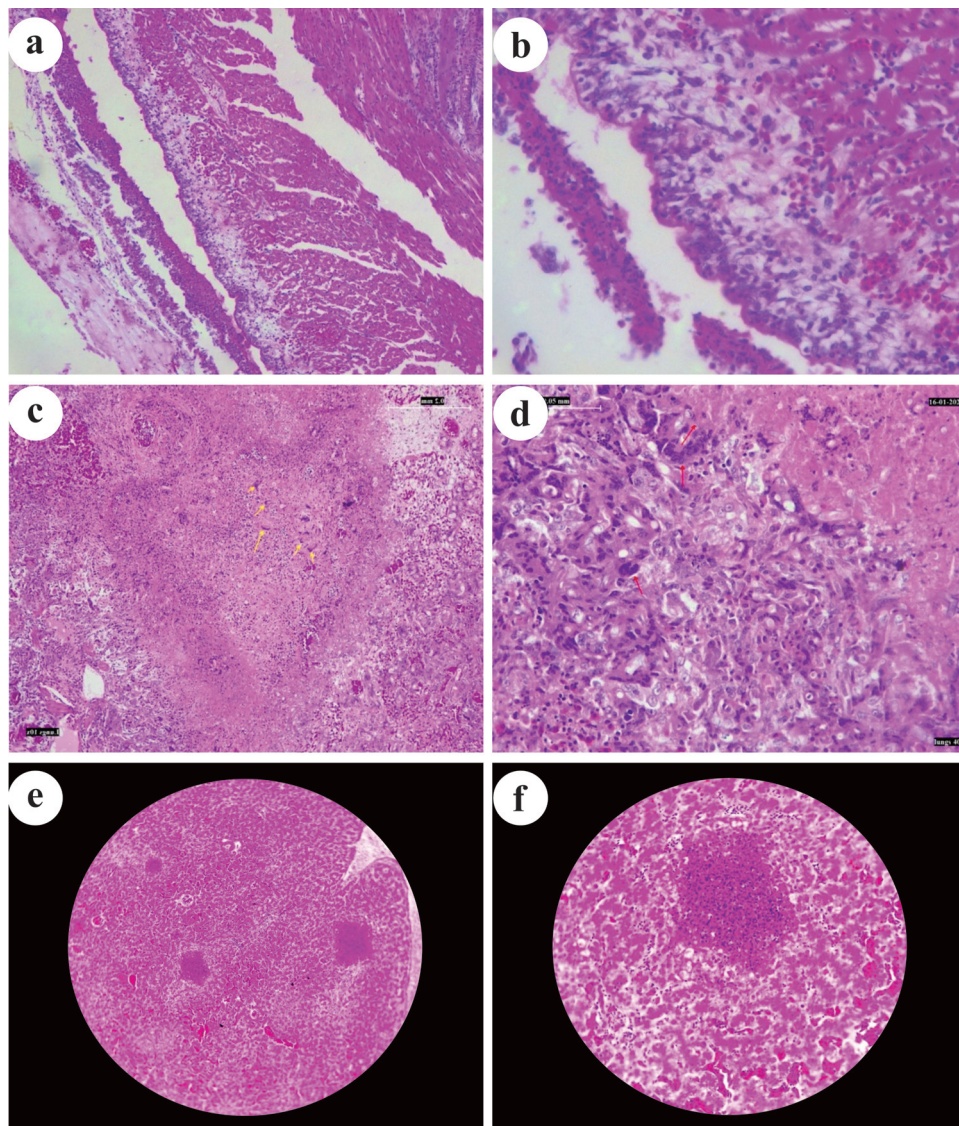


Fig 2a and b. Riemerellosis - section of heart showing fibrinous pericarditis, infiltration of inflammatory cells predominantly heterophils and few mononuclear cells (a) H&E X100, (b) H&E x400. **c and d.** Granulomatous mycotic pneumonia - Lung section showing Multiple foci of necrosis and eosinophilic exudates in alveoli, septate hyphae with heterophilic and lymphocytic infiltration, macrophages and multinucleated giant cells (arrow) surrounding the area of necrosis (c) H&E x100, (d) H&E x400. **e and f.** Pasteurellosis - Liver section showing multiple foci of necrotic foci in the parenchyma with presence of neutrophils and few coccobacillary organisms in the lesion along with diffuse hepatocyte degeneration (e) H&E x200, (f) H&E x400.

which are relatively larger in size than *P. multocida*, indicating the importance of examination of heart blood and impression smears from liver and spleen (Surya *et al.*, 2016). Pillai *et al.* (1993) also noted the size difference of bipolarity between these two organisms. In acute stage of the disease, the organism could be readily isolated from heart blood, liver, spleen, lungs and brain (Pathanasophon *et al.*, 1994; Gooderham, 1996) and ovary (Bisguard, 1995). The diagnosis of the *R. anatipestifer* infection based on clinical signs and symptoms and phenotypic characterization of *R. anatipestifer* and related bacteria such as *P. multocida*, *Salmonella* and *E. coli* by traditional methods is often inconclusive because

of inconsistent reactions and phenotypic diversity (Sandhu, 2008). For the same reason, gene sequencing and phylogenetic analysis is essential to allow proper classification. Molecular detection and characterization of *R. anatipestifer* infection targeting different genes such as *gyrB* gene, *rpoB* gene conserved 16SrRNA gene, Ribonuclease Z gene etc. are highly specific and less time-consuming than the conventional methods (Tsai *et al.*, 2005; Christensen and Bisguard, 2010; Wang *et al.*, 2012; Sarker *et al.*, 2017).

Duck Salmonellosis

Salmonella is a rod-shaped, gram-negative facultative anaerobe that belongs to the family Enterobacteriaceae

(Barlow and Hall, 2002). Within the genus *Salmonella*, two species are there viz., enterica and bongori and around 2600 serotypes have been identified with the use of the standard Kauffman–White scheme and most of these serotypes could adapt within a variety of animal hosts, including humans (Allerberger *et al.*, 2003). Many different serotypes of salmonella have been isolated from ducks, most of these being of public health significance but some, including *S. gallinarum*, *S. pullorum*, *S. typhimurium*, *S. enteritidis* and *S. anatum*, can cause considerable losses in birds of less than a few weeks of age. Affected birds are usually aged between 3 and 12 days, appear to be very depressed and can show signs of scour. True salmonellosis is comparatively rare in ducks but is often due to the serotype *S. typhimurium*. The authors have come across *S. enterica* sub spp *enteritidis* in ducks from flocks belonging to Kerala. Death has been reported to result from a combination of systemic salmonellosis and diarrhoea, the disease being referred to, in the past, as ‘keel’ disease, so-called because birds remained apparently healthy until they keeled over and died. The majority of these isolates are distributed in diseased ducks (57.28%). The relatively high frequency of invasive infection of *Salmonella* in commercial meat-type duck may largely relate to semi-open rearing systems that lack effective biosecurity (Wang *et al.*, 2020). *S. typhimurium* is the only serotype demonstrated in arthritis in ducks. A high incidence of *S. typhimurium* in the intestinal tract of ducks maintained on free range or in open houses suggests that these sites are sources of infection and that the intestinal tract is the portal of entry for *S. typhimurium* causing arthritis (Bisgaard, 1981). Cha *et al.*, (2013) investigated to determine the prevalence at South Korean duck farms and reported that the overall prevalence of *Salmonella* serotypes was 43.4% (69/159) in duck flocks from 65.2% (47/72) of the duck farms. Eighty-five strains were isolated from 69 duck flocks. Three serotypes of *Salmonella enterica* were identified such as *S. typhimurium* (39/85), *S. enteritidis* (44/85), and *S. london* (2/85). The prevalence of salmonella infection decreased significantly in 3-week-old ducks compared to that in 1-week-old ducks. Also, Adzitey *et al.*, (2012) reported an overall prevalence of *Salmonella* serovars as 23.5% (125/531) and the 125 salmonella isolates belong to 10 different serovars namely *Typhimurium* (29.6%), *Enteritidis* (12.0%), *Gallinarum* (2.4%), *Braenderup* (12.0%), *Albany* (11.2%), *Hadar* (20.8%), *Derby* (6.4%), *Wittevreden* (1.6%), *Newbrunswick* (3.4%) and *London* (0.8%). Serovar diversity was the highest in ducks, with the *S. apeyeme* being isolated for the first time from duck tissues. Young ducklings are more likely to become infected by low numbers of salmonella organisms (Henry, 2000). *Salmonella* is relatively resistant to cleaning and disinfection procedures compared with most avian pathogens, and can survive for long periods

of time in residual organic matter in poultry houses (Andino and Hanning, 2015).

S. typhimurium is described as the cause of morbidity and mortality in ducklings, especially during the first 2 weeks of life. A high level of resistance to systemic disease and diarrhea is observed if it exists as a separate clinical entity as no lesions were observed at post-mortem examination and the organism was not recovered from the liver indicating either poor invasiveness and or poor survival of the strain in the reticuloendothelial system as demonstrated in studies. Circumstantial evidence suggests that there is little systemic salmonellosis in the field in very young ducks but that some occurs after cold stress, with the appearance of characteristic lesions. Clinical infections in commercial flocks usually follow a breakdown in hygiene measures on the breeder farm or at the hatchery as described for *E. coli*. Mortality levels in a flock can be quite significant, sometimes reaching 15%. Routine sampling of flocks can reveal quite high levels of salmonella infections with no clinical signs evident. The bacteria and their endotoxins seem to play a significant role in the pathogenesis of amyloidosis in ducks (Ling, 1991).

Post mortem signs include acute dehydration, septicaemia, acute enteritis and the classical caecal cores of white caseous material. Histopathologically, various experimental studies demonstrated congestion in all blood vessels of the most infected groups together with recent thrombus in portal veins of birds. Degeneration and necrosis with variable degree in addition, inflammatory cells infiltration in different organs of the infected groups were observed. *S. gallinarum* could be isolated from intestinal wall samples in densities greater than those found in the lumen, indicating active invasion as has been described for highly invasive salmonella organisms. Diagnosis is by culture and identification of organism and further confirmation by PCR.

Streptococcal and Enterococcal infections

Streptococcus zooepidemicus infection is characterized by increased levels of mortality at around 10- 14 days of age in commercial flocks. *Enterococcus cecorum* (EC) which was considered as a neglected bacterium has been designated as an emerging avian pathogen. EC associated disease outbreaks were also reported from ducks in Germany (Metzner *et al.*, 2010; Jung *et al.*, 2012; 2013). EC which was initially considered harmless commensal of the gastrointestinal tract of birds, have now become a pathogen capable of causing significant morbidity and mortality in birds (Jung *et al.*, 2018). Gaier *et al* (2016) reported that, Enterococci are widely distributed in nature and are not generally recognized as safe. On the contrary, Moellering (1992) and Leclercq and Courvalin (1997) showed that, *Enterococcus faecalis* is a bacterium with low pathogenic potential.

The *Enterococcus* flora of duck intestines consists of *E. faecalis* and *E. faecium* in ducklings less than eight weeks old and *E. faecalis*, *E. faecium* and *E. gallinarum* in ducks more than eight weeks old (Saikika et al., 1994). Among 53 enterococcal species, *E. faecalis*, *E. cecorum*, *E. hirae*, *E. faecium* and *E. durans* have the most crucial role in avian pathology. The authors are skeptical of the role of *E. durans* as a pathogen in ducks as the same was isolated mostly in association with some other bacteria like *E. coli*, *S. aureus* or even *E. faecalis-faecium*. The mean age at the time of isolation of EC was 3.6 weeks in ducks (Dolka et al., 2017). *Enterococcus faecalis* was isolated at a rate of 77.7% and 36.3% from duckling farms and backyard respectively rather than grower ducks. Saikia et al., (1994) isolated *Enterococcus faecalis* (70.9%) from duckling less than eight weeks. Smyth and McNamee (2001) demonstrated that, *E. faecalis*, *E. hirae*, and *E. durans* were the most common enterococcus spp causing both septicaemia and localized infections in poultry. Olsen et al., (2012) reported that *Enterococcus faecalis* was the most significant bacterial pathogens associated with first week mortality in poultry.

Enterococcus sp. can result in distinct clinical forms of disease, acute and subacute/chronic. In the acute form, clinical signs are related to septicaemia and include depression, lethargy, pale combs and wattles, anorexia, reduced water and feed consumption, ruffled feathers, body weight loss, inactivity, diarrhoea, mild head tremors and often, only dead birds are found. In the subacute/chronic form, depression, loss of body weight, lameness and nervous manifestations such as head tremors may be observed. Clinically affected birds eventually die if not treated (Joan and McNamee, 2002). *E. faecalis*, *E. faecium*, *E. durans*, *E. cecorum* and *E. hirae* have occasionally been associated with a variety of pathologies including endocarditis, septicaemia, central nervous disorders and lameness (Wood et al., 2002). *Enterococcus* spp (*faecalis*, *durans*, *hirae*) were isolated at a rate of 52.5% from birds with the ascites syndrome of Ismailia province (Mohamed and Sohair, 2008). Sudden death of some birds was recorded.

EC infection was successfully reproduced experimentally in Pekin ducks (Jung et al., 2013). Serotype 2 was more prevalent in broiler and Pekin duck isolates than serotype 1. Post mortem findings include severe congestion in all internal organs, muscles, ribs, brain, intestine, enlarged spleens which are mottled in appearance, fibrinous exudate in spleen, congestion, haemorrhage, pale liver with necrosis, renal interstitial vascular congestion pericarditis, perihepatitis, ascites, enteritis, hydropericardium, arthritis (amyloid deposition), necrosis of bone extremities with femoral head necrosis, bone abnormalities, arthritis, air sacculitis with the presence of haemorrhagic streaks on muscles

and brain congestion. (Zhang and Han., 2010). The cause of air sacculitis in this instance has not been ascertained. *Enterococcus faecalis* has been described as a cause of omphalitis, endocarditis, meningitis, fibrinous arthritis and/or tenosynovitis.

In experimentally infected birds, a more acute course of disease with generalized bacteremia was detected especially in the second week of life. Yolk sac infection was observed in Pekin ducks as well as ovary infections in Pekin duck breeders but no lesions of the skeletal system such as osteomyelitis were observed (De Herdt et al., 2009; Stalker et al., 2010; Metzner et al., 2010; Martin et al., 2011; Jung et al., 2012). Presence of bloody fluids in cecal core, mild bone abnormalities (necrosis in bone extremities, femoral head necrosis) was detected in intra nasal inoculated ducks.

Histopathological changes include pneumonia with fibrinous exudates in distended blood vessels, follicular hyperplasia, presence of clumps of bacteria and accumulation of fibrinous exudate were detected within the spleen tissue, perivascular accumulation of lymphocytes, monocytic cells and heterophilic granulocytes in the liver. Diagnosis is based on clinical signs, lesions and culture and microbiological identification and further confirmation by molecular diagnostic methods like PCR. Novobiocin has been found to be efficacious in ducks with *E. faecium* infection (Sander et al., 1998). Pathogenic *E. cecorum* isolates are sensitive to penicillin, have high level resistance to macrolide and aminoglycoside antibiotics (Borst et al., 2012)

Staphylococcal infections

Staphylococcus organisms present itself as a part of normal flora of mucous membranes and are widely present in poultry rearing environments. It is a gram-positive bacterium that affects a wide range of avian species which possess several pathogenicity markers, including a clumping factor and Protein A that correlates with clinical cases of staphylococcosis in avian species. Bacterial cell surface protein, Protein A can bind to the fragment crystallizable region (FC) of immunoglobulin and inhibit the phagocytosis by bacteria. The incubation period varies from 48-72 hours. Clinical signs varies from fever, depression, listlessness, ruffled feathers, lameness, reluctance to walk, drooping wings, and sometimes swollen joints (Dias and Mantau, 1994). Outbreak of omphalitis was reported in the week old ducklings (Sharma and Kaushik, 1986) with clinical signs such as swollen abdomen that contains cheesy caseous content into yolk sac, oedema, redness, few cases of peritonitis, septicemia, reduction in feed and water consumption and hence a severely dehydrated carcass (Mondal and Sahoo, 2014). Occasionally, this organism causes pericarditis (Bisgaard et al., 1995), granulomas in liver and lungs, swelling of the testis (Andreasen, 2013) etc. In ducks,

S. aureus was most prevalently isolated from hip joints and joints of toe (Bisgaard, 1981). In turkeys, Green liver – Osteomyelitis complex has been identified as a result of Staphylococcal infection (Bayyari *et al.*, 1994). Fifty percentage of *Staphylococcus aureus* strains produce enterotoxins, some of which cause disease in human as well (Harvey *et al.*, 1982). Organisms could be isolated and identified by culture and molecular diagnostic tools like PCR (Eid *et al.*, 2019). Even though *S. aureus* is the primary pathogen, in few cases, the authors have come across different coagulase negative staphylococci in yolk sac infections in ducks, the clinical significance of which is not clear.

Many broad spectrum antibiotics have been successfully used to treat staphylococcal infection in ducks viz. penicillins, streptomycin, tetracyclines, erythromycin etc. Treatment of some localized lesions is often difficult, due to poor penetration by orally administered antibiotics (Dowling and Kruth. 2006).

Duck Aspergillosis

Aspergillosis is a common, non-contagious fungal infection of ducks caused by members of the fungal genus *Aspergillus*. *Aspergillus fumigatus* is the most common species isolated from infected ducks (Khosravi *et al.*, 2013). Poor ventilation, overcrowding, raising in damp areas with high humidity etc. predisposes bird to this fungal disease (Dutta *et al.*, 2021). Healthy ducks are regularly exposed to fungal spores on a daily basis, sometimes even carrying them in their lungs and air sacs without causing a problem. However, *Aspergillus* spp. are quite opportunistic, so if a vulnerable duck is exposed (usually the result of a concurrent infection or injury that has worn down their immune defenses), then it takes the opportunity to cause infection. Clinical signs depend on the organ affected and varies from mild respiratory distress to severe laboured breathing, listlessness, loss of body weight etc. (Pascal *et al.*, 2011). Aspergillosis can be either localized or systematic, and often results in a progressive, debilitating illness. There are contradictory results as well reported on the prevalence of clinical signs. Ambily and Mini (2017) reported no clinical signs in a Japanese quail farm in India. The primary target of the fungus is the respiratory pathways, including the air sacs. Sometimes it evades the lungs and is limited only to the abdominal air sacs. Sometimes from the abdominal air sacs, the pathogen spreads systemically to various organs like spleen, heart, lungs etc. (Dutta *et al.*, 2021). Intestinal aspergillosis has been reported more in free flying birds than in domesticated poultry. Aspergillosis usually affects the duck's upper and lower respiratory tract, although any organ systems can become infected.

Lesions varies from congestion of the lungs in early stages to presence of multiple nodules in lungs, air sacs, sometimes along with growth of green coloured

velvet fungal colonies in air sacs (Kaboudi *et al.*, 2018). In systemic aspergillosis, the lesions can extend to other visceral organs like liver, spleen, intestine and even brain. Microscopically, focal granulomatous reaction was observed in the lungs with caseous necrotic cellular debris surrounded by rims of heterophils, lymphocytes, macrophages, epithelioid cells and multinucleated giant cells (Chu *et al.*, 2012). Copper Sulphate at the rate of 1g/litre of drinking water has been successfully tried along with antifungals like griseofulvin or fluconazole for 3-7 days for the treatment of aspergillosis (Dutta *et al.*, 2021).

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Newcastle disease vaccines: The current understanding

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ABSTRACT

Newcastle Disease (ND) is a significant viral threat to the global poultry industry, necessitating robust vaccination strategies for disease prevention and control. Vaccination against NDV primarily aims to minimize mortality, clinical disease, and viral shedding while reducing economic losses associated with the disease. Conventional approaches encompass inactivated and live vaccines, each with distinct advantages and limitations. Inactivated vaccines are safe but often less effective in stimulating cell-mediated immunity and can result in viral shedding upon challenge. Live vaccines, cost-effective and efficient in stimulating immunity but with safety concerns such as virulence reversion. Recent advancements in vaccine technology involve recombinant vaccines that present immunogenic fragments, genetically engineered vaccines, vectored vaccines, sub-unit vaccines, virus-like particles (VLPs), immune complex vaccines, cytokine-expressing vaccines, and bacterium-like particles (BLPs). These novel approaches aim to overcome the limitations of conventional vaccines, enhance efficacy, and induce broader and long-lasting immunity. However, challenges such as vaccine failure, evolving viral strains, interference from maternal antibodies, and sub-optimal vaccination practices lead to outbreaks and economic losses. Genotype-matched vaccination and antigen camouflage-antigen decoy strategies for vectored vaccines are promising for overcoming maternal antibody interference. Strict quality control, improved biosecurity, surveillance, and commercialization of indigenously developed recombinant vaccines are vital steps in controlling ND outbreaks. Future ND vaccine development should prioritize rapid adaptability to emerging strains, multiplexed formulations, and region-specific needs to ensure effective disease control across diverse poultry production systems.

Keywords: Genotype matched vaccines, Newcastle disease, Recombinant vaccines

INTRODUCTION

In India, the poultry industry has experienced a significant transformation in the last decade, with an increase in both unorganized backyard poultry and a highly organized commercial sector, which was reflected in the 20th livestock census. The poultry population is about 851.81 million, of which the backyard and commercial poultry account for 317.07 million and 534.74 million respectively. It increased by 45.78 percent and 4.5 percent in the backyard and commercial sectors, respectively, thereby contributing to an overall increase of 16.81 %. Egg production was approximately 129.6 billion with a compound annual growth rate (CAGR) of 6.19 % and meat production was 9.29 million tonnes with a CAGR of 5.62 percent during 2021-22 as per Department of Animal Husbandry and Dairying (DAHD) reports. The poultry sector contributes approximately Rs.1.3 lakh crores to India's Gross domestic product (GDP). According to Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) 2021 reports, India ranked 3rd in egg production and 8th in poultry meat production globally. The poultry industry is expected to grow at the rate of 8-10 percent in 2023-24.

Despite the rapid growth rate, the poultry industry faces many challenges such as threats due to climate change, rise in feed and maintenance costs, and outbreaks due to emerging and reemerging diseases, etc. These challenges not only incur economic losses but also pose a severe threat to food security, which is a public health concern. Climate change is a problem to be addressed at the global level. Feed and maintenance rates can be solved via state and national policies, whereas disease outbreaks can be controlled at the farm level to curb economic losses.

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Outbreaks due to viral diseases are one of the leading causes of economic loss in the global poultry industry (Brown *et al.*, 2018). Major viral diseases causing significant economic losses to poultry include avian influenza (AI), Newcastle disease (ND), Marek's disease (MD), infectious laryngotracheitis (ILT), infectious bursal disease (IBD), and infectious bronchitis (IB). These viral diseases exert a range of adverse effects on poultry, including reduced feed intake, poor feed conversion efficiency, decreased body weight gain, and poor egg and meat quality, causing significant economic losses for both backyard and commercial poultry producers (Roth and Sandbulte, 2021). As

most avian viral diseases are not cured, control measures such as vaccination, surveillance, physical separation, and preemptive culling of infected birds are most commonly employed to prevent the spread of the disease, of which vaccination is the most cost-effective and primary preventive measure recommended by authorities worldwide (Ravikumar *et al.*, 2022).

Though few attempts have been made to estimate the economic losses in India, Newcastle disease virus (NDV) is the leading cause of economic losses in commercial and backyard poultry in India. The majority of the economic loss is mainly attributed to the mortality caused by the disease followed by a drop in production. Despite mass vaccination programs using several vaccines, ND remains a potential threat across the country as well as the globe.

This paper provides an overview of the existing ND vaccines available within the country and discusses their comparative efficacy in controlling ND outbreaks and future perspectives.

NEWCASTLE DISEASE

Newcastle disease, also referred to as Ranikhet disease, pseudo-fowl pest, avian pest, pseudovogel-pest, atypische geflügelpest, avian distemper, Korean fowl plague, Tetelo disease, pseudo-poultry plague, and avian pneumoencephalitis, is a highly pathogenic viral disease that affects more than 200 avian species, with a mortality rate of up to 100% in unvaccinated birds (Suarez *et al.*, 2020; WOA, 2023). The disease was first reported in 1926 in Java, Indonesia (Kraneveld, 1926), followed by an outbreak in Newcastle upon Tyne, in the United Kingdom (Doyle, 1927). The disease is endemic in Asia, Africa, the Middle East, Europe (Poland, France, Norway, Sweden, and Belgium), and Central and South America (WOA, 2023), resulting in major economic losses to the poultry industry in developing countries.

In India, ND was first reported in Ranikhet, Uttarakhand in 1928 (Edwards 1928), Chennai (Madras), Tamil Nadu in 1930 (Kylasamaier 1931). Since then, ND has become endemic to the country with outbreaks occurring regularly in both commercial and backyard poultry (Gowthaman *et al.*, 2019; Joshi *et al.*, 2021; Puro and Sen 2022; Ravikumar *et al.*, 2022; Sahoo *et al.*, 2022;; Rajkhowa *et al.*, 2023).

TAXONOMY AND STRUCTURE

Newcastle disease is caused by the NDV which is avian paramyxovirus type 1 (APMV-1), and belongs to the genus *Orthoavulavirus* (formerly *Avian Avulavirus*), sub-family *Avulavirinae*, family *Paramyxoviridae*. APMV-1 or NDV affects both domestic and wild birds, but the severity of the disease depends on the host and virus strains (Amarasinghe, 2019; Dimitrov *et al.*, 2019).

Virions exhibit marked pleomorphism, often adopting a spherical morphology with a diameter ranging between 100 and 500 nm or a filamentous shape with a diameter of approximately 100 nm. The viral nuclear core is characterized by a singular left-helical symmetrical nucleocapsid enveloped by a bilayer lipid membrane derived from the host. This lipid envelope projects two viral surface glycoprotein spikes, comprising hemagglutinin-neuraminidase and a fusion protein, measuring 8-12 nm in length (Nagai 1989; Alexander 2001; Yusoff and Tan, 2001; Mao *et al.*, 2022).

NDV is a negative sense, single-stranded, non-segmented RNA virus. Three different full-length genomes of sizes 15,186 bp, 15,192 bp, and 15,198 bp are seen in NDV (Czeglédi *et al.*, 2006; Dimitrov *et al.*, 2017; Bello *et al.*, 2020; Mao *et al.*, 2022). The genome contains six non-overlapping genes that encode structural proteins, namely nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large RNA-dependent RNA polymerase (L) with a 55-nucleotide extra cistronic leader sequence at the 3' end and a 114-nucleotide trailer sequence at the 5' end, which play a vital role in viral transcription and replication. The order of genes is 3' leader - NP-P-M-F0-HN-L-5'trailer (Nagai, 1989; Suarez *et al.*, 2020). Additional non-structural proteins such as V and W can also be produced by RNA editing of the P gene, (Brown and Bevins, 2017; Suarez *et al.*, 2020).

GENOTYPES

NDV is broadly classified into two classes I and II, class I comprises of single genotype and three sub-genotypes namely 1.1.1, 1.1.2, and 1.1.3. They are prevalent in wild birds and most of them are lentogenic in nature and used as oncolytic viruses or recombinant vaccine vectors (Schirrmacher, 2016). Class II comprises of the pathogenic genotypes circulating among the poultry and wild birds. They are phylogenetically classified into 21 genotypes (I - XXI) with diverse sub-genotypes and NDV vaccines are targeted against these genotypes (Dimitrov *et al.*, 2019). The broad host range and widespread distribution of the NDV, aided by wild reservoirs, allows it to undergo continuous evolution, leading to the emergence of distinct genotypes every year in various geographical locations (Miller *et al.*, 2009, 2010). Given the emergence of new strains worldwide, it has become imperative to classify NDV-genotypes to comprehend their distinct characteristics and assess their potential impacts on animal health. Furthermore, it facilitates the use of an appropriate circulating strain for vaccine production to control the disease (Ballagi-Pordany *et al.*, 1996).

Previously, the virus was characterized using pathogenicity tests like ICPI and IVPI (Allan *et al.*, 1978),

neutralization tests (Bankowski and Kinjo, 1965), plaque formation assay (Takehara *et al.*, 1987), and hemagglutination inhibition tests using monoclonal antibodies (Russell and Alexander, 1983; Alexander *et al.*, 1987). With the advancement of molecular techniques, the classification has been modified based on the HN gene as A, B, and C lineages (Sakaguchi *et al.*, 1989), Restriction Fragment Length Polymorphism (RFLP) analysis of F gene into genotypes (I-VI) (Ballagi-Pordany *et al.*, 1996), partial nucleotide analysis of F gene (47-420 nt) of NDV isolates resulted in two major classes - Class I (15,192 nt) and Class II (15,186 and 15,198 nt) into lineages and sub-lineages (Aldous *et al.*, 2003; Czeplédi *et al.*, 2006; Dimitrov *et al.*, 2019).

Different classification systems without well-defined criteria have created discrepancies in the classification of new isolates. Diel and co-workers proposed an unbiased classification system based on the complete F gene sequence to resolve the difficulties of prior classification systems (Diel *et al.*, 2012). The widespread distribution and rapid evolution of NDV have impeded its effective classification. Additionally, the classification of new genotypes using partial F gene sequences without adherence to establish criteria has resulted in inconsistencies in the classification. An international consortium comprising 27 Office International des Epizooties (OIE) reference NDV laboratories and global scientists was established and three working groups were formed to revise the existing classification system and establish a unified classification framework with updated guidelines for data collection, curation, classification, naming, and open access dissemination of partial and complete curated data (Dimitrov *et al.*, 2019). The revised classification system employs phylogenetic topology and evolutionary distances strategy to categorize isolates into novel genotypes or sub-genotypes. The establishment of new genotypes or sub-genotypes is contingent upon the availability of at least four distinct isolates. The assignment of viruses to newly defined genotypes and sub-genotypes is solely based on a comprehensive dataset of sequences encompassing all known genotypes. Distinct genotypes exhibit an average distance per site exceeding 10% (0.1), while distinct sub-genotypes exhibit an average distance per site exceeding 5% (0.05). The system utilizes nomenclature criteria and also preserves all existing genotypes, retaining their original Arabic or Roman numeral designations. Class 1 genotypes are designated using Arabic numerals (e.g. Genotype 1) whereas class 2 genotypes are represented using Roman numerals (e.g. Genotype I, II, etc.); sub-genotypes are assigned names using a numerical-decimal system, commencing with the genotype's numeral followed by decimal numbers separated by periods (1.1). At each defining node in the phylogenetic tree, the dichotomous splitting system is

employed to name sub-genotypes (e.g. Class I - sub-genotype 1.1 and class II - sub-genotype II.1, II.2). Newly discovered viral diversity that adheres to all classification criteria is classified as a novel genotype and receives a subsequent Roman numeral (e.g. Genotype XX). Existing sub-genotypes that satisfy the genotype criteria retain their original names. This novel classification system addresses the challenges of its previous system, offering a more consistent and informative approach to naming NDV genotypes and sub-genotypes. When a new sub-genotype of viruses is discovered later but still belongs to a higher order within an existing genotype, it is assigned the next consecutive numerical address corresponding to its respective order level in the phylogeny. This approach prevents the need to renumber all existing sub-genotypes of higher orders. For instance, if a new sub-genotype is identified that outgroups V.1.1 and V.1.2 but still falls within V.1, it would be named V.1.3 (Dimitrov *et al.*, 2019). Genotype XII.1 (XII.1.2.2) was found to be the current circulating genotype responsible for the outbreak (Gowthaman *et al.*, 2019). Novel genotypes XXII.1 and XXII.2 were found to be prevalent circulating strains in North-East India, (Rajkhowa *et al.*, 2023).

PATHOTYPES

Based on the severity of the infection the NDV strains are grouped into five pathotypes such as viscerotropic velogenic form which is a high pathogenic form that causes hemorrhagic intestinal lesions; neurotropic velogenic form which causes high mortality with predominant respiratory and nervous signs; mesogenic form that produces low mortality with respiratory signs accompanied with occasional nervous signs; lentogenic form is characterized by mild or subclinical respiratory infection and sub-clinical or asymptomatic form with sub-clinical enteric infection (Gallili and Ben-Nathan 1998; Alexander 2001; WOA, 2023).

The intracerebral pathogenicity index (ICPI) serves as an alternative method for assessing virulence, distinct from molecular techniques involving RT-PCR and sequencing. APMV-1 isolates displaying an ICPI of ≥ 0.7 , or those featuring multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 of its F protein, are classified as virulent (WOA, 2023).

The current outbreak causing genotype XII.1 and genotype XXII serotypes are velogenic in nature resulting in clinical signs which are predominantly neurotropic with a mortality rate of 90-100%.

NEWCASTLE DISEASE VACCINES

Immunization against NDV-inactivated or live ND vaccine is the most widely implemented preventive

Table 1. List of vaccine strains used by different companies in India (Approved by Central Drugs Standard Control Organisation).

S.No.	Name of the Manufacturer	Name of the Strain	Source of the Vaccine
1.	Globion India Pvt. Ltd.	ND Virus, LaSota Strain	IVRI, Izatnagar, Uttar Pradesh (UP)
		ND Virus, F Strain	IVRI, Izatnagar, U. P
2.	M/s. Virbac Animal Health India Pvt. Ltd	ND Virus, TANUVAS D58 Strain	Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai
		Newcastle disease virus (KBNP-C4152R ₂ L Strain)	Not Known
3.	Zydus Animal Health & Investments Ltd	VH Strain	Kimron Veterinary Institute, Israel
4.	M/s. Boehringer Ingelheim India Pvt. Ltd.	VG/GA - Avinew Strain	Boehringer Ingelheim
		ND virus, LaSota Strain	Boehringer Ingelheim
5.	Venkateshwara Hatcheries Pvt. Ltd. (VentriBiologicals, Vaccine Division)	ND virus, LaSota Strain	M/S Tri-Bio Laboratories Inc, USA
		ND virus, R ₂ B Strain	IVRI, Izatnagar, U.P.
6.	HIPRA India Pvt. Ltd.	ND virus, Clone CL/79	Not Known
7.	Hester Biosciences Ltd.	ND virus, B1 strain	Maine Biological Laboratories, Maine State, USA
		ND virus, LaSota Strain	Maine Biological Laboratories, Maine State, USA.
8.	M/s Indovax Pvt. Ltd.	ND virus, R ₂ B Strain	IVRI, Izatnagar, U.P.
		ND virus, F Strain	IVRI, Izatnagar, U.P.
		ND virus, LaSota Strain	IVRI, Izatnagar, U.P.
		ND virus, R ₂ B Strain	IVRI, Izatnagar, U.P.
9.	Intervet India Pvt. Ltd.	ND virus, Clone 30	Not Known
10.	M/s Ceva Polchem Pvt. Ltd.	ND virus-SZ LaSota strain	Newcastle disease virus strain designated as "SZ", obtained from Syria, with an intermediate of Dr Vilmos Palya
11.	Vaksindo Animal Health Private Limited	ND Virus Genotype VII of N018 strain	ND virus Genotype VII strain N018 is a local isolate from India
12.		ND Virus Ulster strain	The master seed virus Newcastle disease of Ulster the strain originated from the U.S.A.
13.	Stallen South Asia Pvt Ltd.	ND Virus, LaSota Strain	Owned by the manufacturer i.e. FATRO S.p.A, Italy
14.	Zoetis India Limited	NDV Kimber strain	Master seeds used were acquired as part of the acquisition on March 1, 1997, of Solvay Animal Health, Est. No. 195 by Fort Dodge Animal Health, Est. 112. The master seed was subsequently acquired as part of the acquisition of Wyeth Corporation and its subsidiary, Fort Dodge Animal Health, Est. 112 on October 16, 2009, by Pfizer Animal Health, Est. No. 189. Pfizer Animal Health, Est. 189 transferred all master seeds to Zoetis Inc., Est. 190 on March 28, 2013.
15.	M/s Elanco India Pvt. Ltd	ND Virus, LaSota Strain	Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin

measure in poultry production worldwide (Miller *et al.*, 2007). Vaccination against ND primarily aims to reduce mortality, prevent clinical disease, minimize the shedding of highly pathogenic NDV, and reduce the economic loss associated with the disease as much as possible (Senne *et al.*, 2004; Kapczynski *et al.*, 2013; Dimitrov *et al.*, 2017). Conventional vaccines using killed or inactivated forms, and live viruses (naturally occurring or attenuated) are intensively used in the field. Currently, novel genetically engineered vaccines that present only an immunogenic fragment of the pathogen, inducing immunity without any side effects are also employed (Senne *et al.*, 2004; Miller *et al.*, 2007; Tizard, 2020; Hu *et al.*, 2022).

Inactivated or killed vaccines

Inactivated vaccines serve as a preventive measure against ND. Lentogenic strains such as LaSota and B1 or mesogenic strains like R2B and Ranikhet disease vaccine K (RDVK) are inactivated using formalin, binary ethylenimine or beta propiolactone. These vaccines are often emulsified with adjuvants like mineral oil to enhance their efficacy. The primary advantage of inactivated vaccines relies in their heightened safety profile, stemming from their inability to replicate or disseminate among avian populations. Consequently, maternal antibodies do not impede the effectiveness of the vaccine. Typically, these vaccines are administered in older birds via parenteral route such as intramuscular or subcutaneous which is laborious, and incurs higher production costs due to the necessity for a higher dosage to elicit immunity. While they provoke a robust humoral immune response, they often fall short in stimulating cell-mediated immunity. Additionally, upon challenge, vaccinated birds may shed virulent viruses (Dimitrov *et al.*, 2017; Suarez *et al.*, 2020; Hu *et al.*, 2022).

Live Vaccines

Live ND vaccines are widely employed for preventing clinical disease due to their cost-effectiveness and efficacy in stimulating immunity. Identical antigenic property of the strains allows their usage globally. The most common lentogenic strains utilized as live vaccines are LaSota, B1, and F. These vaccines can be administered through various routes, including ocular drops, oral drops, spray, and in drinking water. They are cultivated in embryonated eggs or tissue culture, and these vaccines are stored in lyophilized form under cold conditions (Gallili and Ben-Nathan, 1998; Suarez *et al.*, 2020).

Live vaccines can stimulate IgA production

thereby inducing mucosal immunity in addition to the humoral, and cellular immune responses (Miller *et al.*, 2009; Suarez *et al.*, 2020). The potency of live ND vaccines is reliant on the dosage administered; for instance, for LaSota - though an EID (egg infective dose) of 10^4 – 10^5 can control mortality, it cannot prevent the clinical disease and virus shedding on challenge and requires a minimum EID of 10^6 to elicit robust immunity and diminish viral shedding. However, this requirement might lead to elevated expenses in disease control practices (Cornax *et al.*, 2012; Dimitrov *et al.*, 2017). Mesogenic strains like Mukteswar and RDVK possess high immunogenicity, facilitating rapid and long-lasting immune responses. Yet, their use may result in mortality, thereby they are typically employed as boosters solely in adult birds within endemic regions (Hu *et al.*, 2022). Additionally, thermostable strains such as I2 are deployed in vaccinations, where maintaining a cold chain significantly influences vaccine effectiveness (Dimitrov *et al.*, 2017).

In India, lentogenic strains like F and LaSota are widely used because of high tropism and replicative ability whereas mesogenic strains like R₂B and RDVK are used in combination with lentogenic strains in virulent strain endemic regions (Table 1). The vaccination schedule (for commercial layers) followed widely in India is represented in the table below (Table 2).

Recombinant vaccines

Genetic engineering via recombinant DNA techniques aims to address the limitations of conventional vaccines. Recombinant vaccines employ various reverse genetics strategies such as genetically modifying pathogens and rendering them avirulent yet maintaining their immunogenicity, identifying immunogenic antigens within the pathogen, and utilizing diverse delivery strategies to administer these antigens to produce protective immunity. Recombinant vaccines can be used to differentiate infected and vaccinated animals by DIVA strategy over conventional vaccines. By in-silico analysis, different potential antigens of different strains of the same pathogens or different pathogens can be genetically fused to produce a chimeric vaccine which makes it an attractive veterinary vaccine.

Genetically modified vaccines

By reverse genetics approach various pathogens are genetically modified to reduce their pathogenicity while preserving their immunogenic potential. Several lentogenic and mesogenic strains are genetically reconstructed to produce modified strains with enhanced efficacy. Yadav *et al.*, 2018 genetically modified Mukteswar (R₂B), mesogenic strain into a lentogenic strain while retaining its ability to produce protective immunity identical to the original mesogenic strain, so that it can

Table 2. Vaccination schedule (Commercial layers).

Age	Vaccine strain	Route
5-7 days old chick	F / LaSota	Eye / Nostril
35 days	F / LaSota	Eye / Nostril / Drinking water
8-10 w	R ₂ B/RDVK strain	SC / IM
16-18 w	R ₂ B/RDVK strain	SC / IM

be used in primary vaccination programs which is not feasible with conventional R₂B strain.

Vectored Vaccines

Vectored vaccines are recombinant vaccines that employ a suitable vector, comprising viruses or bacteria, to deliver foreign antigens or vaccine genes into the target animal. These vectors are genetically engineered by incorporating one or more specific genes that code for the targeted antigens. Vectored vaccines stimulate both humoral and cell-mediated immunity (CMI). They ensure safety, offer diverse administration routes, possess inherent adjuvant properties, and the option to render them either replication-competent or incompetent to minimize adverse reactions. Viral vectors like herpesvirus of turkey (HVT), adenovirus, poxvirus, and Newcastle disease virus are widely used in veterinary, (Jorge and Dellagostin, 2017).

Vectored ND vaccines mainly utilize genes that encode for F or HN proteins. HVT, one of the most widely used vectors utilizes the F gene of NDV and has been shown to offer protection against both Newcastle disease and Marek’s disease. It is usually administered via *in-ovo* route in 18-19 days embryonated eggs and *subcutaneous* route in one-day-old chicks. Palya *et al.*, (2012) demonstrated that it requires a minimum of 28 days for the development of complete immunity against NDV. It has been also shown that HVT-based trivalent vaccine harbouring NDV and IBD immunogenic genes produced robust immunity in the presence of maternal immunity without affecting the hatchability under field conditions (van Hulten *et al.*, 2021). HVT vectored vaccines offer mass administration at the hatchery level, induce both humoral and CMI, and provide long lasting immunity due to persistent nature of HVT (Hein *et al.*, 2021; Hu and Liu 2021). Currently, four HVT-based ND vaccines are available for commercial use.

HN and F proteins of NDV expressed via recombinant Fowl pox virus (rFPV) was capable of producing immunity against ND and FP as demonstrated by Boursnell *et al.*, (1990). Currently, it is not widely employed because of its decreased efficacy due to prior exposure to FPV, significant interference by maternal antibodies (MDA), and constraints in mass vaccination due to the subcutaneous or wing web stabbing route of administration (Dimitrov *et al.*, 2017; Hu and Liu, 2021). TROVAC-ND is the only currently commercially available rFPV vectored ND vaccine.

Recombinant NDV is capable of expressing foreign genes thereby acting chimeric vaccine by inducing immunity against the target antigen and NDV vector (Zhao and Peeters, 2003). Genetically engineered NDV has a natural predilection towards the respiratory and digestive tracts, thereby inducing heightened mucosal immunity. Further, there is potential to carry heterologous genetic material and efficiently express target antigens, eliciting robust antibody-mediated immunity (AMI) and CMI response. NDV replication occurs in the cytoplasm, thus negating the risk of integrating its genome into the host genomic DNA, a potential concern associated with certain viral vectors. Moreover, rNDV demonstrates minimal genetic recombination, ensuring the stability and safety of the vaccine (Romanutti *et al.*, 2020). NDV has receptors for various animal species including humans, making them one of the promising vectors for vaccine delivery. Many NDV vectored bivalent poultry vaccines have been developed against Infectious bursal disease (Dey *et al.*, 2017), Avian Reovirus infection (Saikia *et al.*, 2019), Highly pathogenic avian influenza (Kim and Samal, 2019), infectious laryngotracheitis virus (ILT) (Basavarajappa *et al.*, 2014) and chicken infectious anemia (Chellappa *et al.*, 2021). Hindrance from maternal antibodies poses a major concern in the

Table 3. Commercially available vectored vaccines for NDV.

S.No.	Name of the Vaccine	Vector	Gene
1	TROVAC NDV	Fowl pox virus	HN and F genes of the Texas strain
2	NEWXXITEK HVT+ND	Recombinant serotype 3 HVT	F gene of Texas strain
3	VAXXITEK HVT+IBD+ND		F gene of NDV genotype VII 1.1 f
4	VECTORMUNE nd	HVT (FC 126)	F gene of NDV D26
5	INNOVAX - ND	Recombinant serotype 3 HVT (PB1 strain)	F gene of NDV clone 30
6	INNOVAX - ND-IBD		
7	INNOVAX - ND SB		
8	INNOVAX - ND-IB-ILT		
9	POULVAC PROCERTA HVT-ND	HVT (FC 126)	F gene of NDV
10	POULVAC PROCERTA HVT-ND-IBD		
11	ULTIFEND™ IBD ND + SB-1	Recombinant serotype 3 HVT	F gene of NDV D26
12	ULTIFEND™ IBD ND		

Modified from Hein *et al.*, (2021)

use of NDV vectored vaccines because they impair the efficacy of the vaccine (Hu *et al.*, 2020). Dimitrov and co-workers developed a novel rNDV vector-based in-ovo vaccine with an antisense sequence of avian interleukin 4 (IL-4) that was able to bypass maternal antibodies and provide full protection from early virulent challenge. The vaccines were administered to 18-day-old embryonated eggs and were found to be safe and effective. At 14 days post-hatch, chicks vaccinated with the rNDV vaccines were fully protected from challenge with a virulent NDV strain (Dimitrov *et al.*, 2021). The different vectored based NDV vaccines are depicted in Table 3.

Subunit vaccines

Subunit vaccines are second-generation vaccines that use the components of the pathogen such as antigenic protein or fragment with high immunogenicity rather than whole organisms. ND subunit vaccines mainly utilizes F or HN or both the proteins. They are expressed using different expression systems such bacteria (*Escherichia coli* and *Bacillus subtilis*), animal cell lines (Chicken embryo fibroblast and Quail fibroblast), insect cell lines (Sf9 and Sf21), plants (corn, potato, and Tobacco) (Lye *et al.*, 2023). Lee and coworkers (2008) compared the immune efficacy of the individual F and HN sub-unit vaccines as well as combined and concluded that the sub-unit vaccine comprising of both HN and F proteins due to their synergistic action offered better protective immunity than the individual sub-unit vaccines after booster (Lee *et al.*, 2008). F protein-based plant-derived sub-unit vaccine was found to offer sufficient protective immunity following booster vaccines in which they induced good mucosal and humoral immunity against virulent NDV without affecting the weight gain of the birds and was also used for differentiating infected and vaccinated animals, (Ma *et al.*, 2020). Recombinant NDV HN subunit also induced sufficient protective immunity after proper downstream processing (Lye *et al.*, 2020). It offers higher safety and thermostability but challenges such as insoluble aggregates formation on expression in recombinant systems, weak immunogenicity and instability in mucosal environments need to be addressed to make it a potential commercial vaccine candidate (Lye *et al.*, 2023).

Smith and his colleagues employed *Nicotiana. Benthamiana* to express F and HN proteins derived from an NDV genotype VII.2. This resulted in the successful generation of VLPs, which were subsequently verified using transmission electron microscopy (TEM). ND VLPs exhibited the ability to bind to sialic acid receptors on chicken cells, as evidenced by their agglutination of chicken erythrocytes. Chickens immunized intramuscularly with partially purified VLPs produced both F- and HN-specific antibodies. The protective efficacy of these findings suggests that

ND VLPs produced in *N. benthamiana* hold promise as effective and safe vaccines against ND (Smith *et al.*, 2023).

Bacterium-like particles

Bacterium-like particles (BLPs) are non-living particles derived from probiotic bacteria that have been engineered to express heterologous antigens. BLPs are effective carriers of antigens for mucosal vaccination (Zhou *et al.*, 2023). A study by Wang *et al.*, (2023) demonstrated the effectiveness of BLPs expressing the F or HN recombinant protein of NDV genotype VII in protecting chickens against challenges with the same virus strain. Intranasal administration of BLPs expressing either the F or HN protein resulted in complete protection, whereas inactivated ND vaccine provided only partial protection. Though the results suggest that BLPs expressing the F or HN protein are promising candidates for a mucosal subunit vaccine against genotype VII NDV, further research is needed to optimize the production and delivery of BLPs-based ND vaccines and to evaluate their efficacy.

Subviral particle or Virus-like Particle vaccine

Virus-like particles (VLPs), mimic the morphology and structure of infectious viruses by assembling into viral structural proteins and lipids without the viral genome maintaining their antigenicity. The non-infectious nature, low potential for recombination, and strong immunogenicity potential make VLPs a promising alternative for viral vaccines (Tan and Jiang, 2014). Wu *et al.*, (2019) showed that chimeric IBD-ND VLPs produced strong humoral immunity comparable to inactivated adjuvant vaccines, stronger cell-mediated immunity (both Th1 and Th2) than inactivated vaccines, and also reduced viral shedding more effectively than inactivated vaccines. ND VLPs has the potential to incorporate glycoproteins from different NDV strains, presenting a possibility for broader protection against various ND viruses. Additionally, these versatile VLPs can be engineered to express protein sequences from diverse pathogens, suggesting extensive applications in vaccine design (Dimitrov *et al.*, 2017). The disadvantage of the VLP vaccine is the complicated purification procedure which increases the cost of the vaccine.

Immune-complex vaccines

Immune complexes or antigen-antibody complexes provide a delayed-release mechanism for the vaccine antigens, ensuring that they are not neutralized by the chick's maternal antibodies until the chick's immune system is mature enough to respond effectively (Jeurissen *et al.*, 1998). Pokric and coworkers (1993) investigated the effectiveness of an immune complex (IC) composed of chicken Newcastle disease virus (NDV) and specific antibodies for protecting chickens against NDV infection. Compared to commercially available vaccines, the IC proved equally effective in generating high levels of

protective antibodies and preventing mortality after a challenge with a virulent NDV strain. Kapczynski *et al.*, (2012) provide evidence that vaccination with a complex of live B1-LaSota NDV and NDV-specific antiserum via the in-ovo route on the 18th day of the embryo can provide complete protection against virulent NDV infections in chicks. They induce better local and humoral immunity however viral shedding was noticed but lower than in case of live vaccines. Further study and optimization are required to establish it commercially.

Cytokine expressing vaccines

Cytokine-expressing vaccines use recombinant viruses as vectors for cytokine expression and these vaccines can induce strong humoral and cellular immune responses, effectively protecting chickens against virulent challenges. Susta *et al.*, (2013) demonstrated that inducing the co-expression of IFN-gamma by vNDV vector resulted in virus neutralization, thereby decreasing morbidity and mortality in specific pathogen free (SPF) chickens. However, Cardenas-Garcia *et al.*, (2015) experiments concluded that regardless of the vaccination system used there was no observed increase in immunogenicity of the evaluated vaccine candidates with cytokine co-expression (Dimitrov *et al.*, 2017). These findings highlight the divergence in outcomes regarding IFN-gamma's influence on the effectiveness of ND vaccines, emphasizing the complexity of immune responses and vaccine interactions in avian models. Further research and development are needed to optimize the cytokines expression levels to balance immune responses between Th1 and Th2, cytokines delivery methods and dosages, ensuring long-term stability and efficacy of the vaccines, and address potential safety concerns for practical application.

The need for vaccination reprogramming

Despite the widespread use of vaccines throughout the country, ND remains a major problem to the poultry industry, causing outbreaks and substantial economic losses. Vaccine failure is mainly attributed to factors like a mismatched vaccine, compromised host defense mechanisms, the hindrance with maternal antibodies, and stress factors (Hu *et al.*, 2022). In endemic areas, the use of low-virulence vaccine strains may inadvertently select virulent strains through immune pressure. Vaccination may protect flocks from overt disease but may not prevent virus shedding. Avirulent NDV strains have the potential to revert to virulence through mutations in the F1 subunit, facilitating cleavage by host furin proteases. This reversion can lead to the spread of virulent NDV within and between farms, even with adequate bio-security measures (Joshi *et al.*, 2021).

Current vaccination is not very effective in India because of the use of outdated vaccine strains, inadequate quality control measures, and improper vaccination

techniques. The present vaccine strains used in India are often derived from older, less virulent NDV strains, rendering them less effective against the circulating virulent strains. Furthermore, reduced quality control practices during vaccine production also contribute to inconsistent vaccine efficacy. Additionally, a lack of standardized vaccination practices and insufficient awareness among poultry farmers regarding proper vaccination techniques hinder effective ND control (Kumar, 2015). Despite many recombinant indigenous vaccine candidates developed, the country has no commercialized recombinant vaccine for field use.

FUTURE APPROACHES

The evolution of NDV due to new mutations in the F-gene, highlights the need to use closely matched vaccines. These vaccines can potentially provide higher survival rates compared to older, conventional vaccines that may not be as effective against emerging strains. Genotype-matched NDV vaccines resulted in lower mortality rates, high protective immunity, and a significant drop in NDV shedding compared to genotype-mismatched vaccines in broilers challenged with velogenic NDV (Kamel and Sultan 2023). The use of genotypically matched inactivated vaccine produced from the endogenous field isolates as booster vaccination following live vaccine provided better immunity against a virulent challenge as they induced a stronger humoral response and reduced virus shedding and may help to control the widespread circulation of velogenic NDV (Hossain *et al.*, 2023, Sultan *et al.*, 2021). Combined Antigen camouflage - antigen decoy strategy can be employed in the case of vectored vaccines to overcome maternally derived antibody (MDA) interference. In the case of vectored vaccines, vectors like NDV are neutralized by binding of MDA with HN and F proteins, as a result, protective immunity is compromised. Antigen camouflage strategy for NDV vector involves the replacement of HN and F genes with antigenically distinct HN and F genes of APMV to produce a chimeric NDV vector that can evade MDA interference whereas the antigen decoy strategy employs co-expression of soluble and membrane-bound HN protein. By integrating these two strategies, a vectored vaccine that can evade MDA with high efficacy can be produced. Though this strategy needs optimization, it has the potential to improve the efficacy of NDV-vectored vaccines and expand their application in poultry vaccination programs (Hu and Liu 2021).

Stricter quality control measures should be implemented to ensure the consistency and efficacy of ND vaccines. Bio-security measures, such as quarantine of new birds and proper disposal of carcasses, can help to prevent the spread of NDV. NDV Surveillance

of the virus in non-outbreak situations could improve scientific understanding of the evolutionary patterns of NDV. Commercialization of indigenously developed recombinant vaccines for field use in outbreaks holds a potential key in controlling ND across the country.

CONCLUSION

ND outbreaks occur globally but the disease situation varies in different countries and poultry farms. ND lurks to be a potential threat to the Indian poultry sector. Vaccination is the most effective method of disease prevention and control. Present vaccination strategies are not fully efficacious in all environments, and new vaccine strategies like genotype matching are needed. Future ND vaccines should be rapidly developed to target emerging strains and allow for multiplexed vaccines. Next-generation ND vaccines should be fit-for-purpose and address the specific needs of different regions and poultry production systems.

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New generation vaccines for economically important poultry viral diseases

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ABSTRACT

Prevention of poultry viral diseases depends mainly on the live attenuated and inactivated vaccines. A vaccine that is highly immunogenic and efficacious with the ability to induce humoral and cell mediated immunity at fewer vaccine doses is considered ideal. Thus, recombinant vaccines stand as a preferred tool for developing poultry vaccines. In the last three decades, recombinant vaccine technology has played an important role in generating innovative poultry vaccines. It combines many advantages over conventional vaccines including safety, efficacy, single hatchery administration applicable by mass vaccination, and the ability to induce a protective immune response in the presence of maternal antibodies. Commercially available recombinant vaccines are subunit vaccines, virus like particles (VLPs), immune complex (Icx) vaccines, and viral vectored vaccines. Current poultry licensed vector vaccines are based on vectors such as Fowl pox, Newcastle disease virus (NDV), and Herpes virus of turkey (HVT), the latter type being the most widely used. With the use of these vectors, many recombinant vectored vaccines have been generated against infectious bursal disease, infectious bronchitis, avian influenza, and infectious laryngotracheitis. The current review focuses on the new generation recombinant vaccines, especially virus vectored vaccines for a better understanding of vaccine safety and efficacy to prevent economically important poultry viral diseases.

Keywords: Poultry, new generation vaccine, viral diseases, recombinant vaccines

INTRODUCTION

In poultry industry, recombinant vaccines can contribute to more effective and safer disease control strategies in poultry farming. They prevent the spread of diseases without causing harm to the vaccinated birds (Romanutti *et al.*, 2020). Economic losses for the global poultry sector are significantly impacted by viral outbreaks making them a primary cause. The prevailing vaccines to prevent these viruses rely on inactivated and live attenuated ones. Though inactivated vaccines are safer when compared to live vaccines, their immunogenicity is low. On the other hand, risk of reversion in live attenuated vaccines and the difficulty in distinguishing vaccinated birds from naturally infected birds has paved the way for the implementation of recombinant vaccines for poultry viral diseases (Ravikumar *et al.*, 2022). Consequently, there is a great demand to implement vaccination strategies that can induce robust and long-lasting sterile immunity against multiple pathogens with a single dose given either *in ovo* or to day old chicks.

Recombinant subunit protein stands out as well-established stable vaccines for Differentiating Infected from Vaccinated Animal (DIVA) compatible options. Complete protection against the disease does not necessarily require the entire pathogen. Subunit vaccines came into a role to minimize the side effects from the vaccine other than the immunogen by fractionating virus proteins. However, subunit vaccines have a few drawbacks which include a complex purification process, low yield, and high production costs. In addition, subunit vaccines have low immunogenicity and requirement of high dosage and booster vaccination. These disadvantages have greatly reduced the use of subunit vaccines for commercial application. Virus Like Particles (VLPs) are multiprotein supramolecular complexes that are structurally similar to their native viral capsid and are devoid of genetic material. VLPs being a recent recombinant vaccine

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candidate, have gained significant scientific interest due to their status as one of the most promising biopharmaceuticals, which led to their usage as safe and effective immunogen characteristics. (Leong *et al.*, 2023). But separation and purification methods for VLPs like precipitation and ultracentrifugation possess drawbacks such as laborious, poor scalability, time consuming, and low productivity.

Immune complex vaccines (Icx) comprise specific antibodies against the virus obtained from hyperimmunized chicken sera combined with the infectious virus (Whitfill *et al.*, 1995). A key advantage of Icx is their applicability for *in-ovo* vaccination

on day 18 of incubation as well as on the 1st day of life of the chick within the hatchery (Ivan *et al.*, 2005). Moreover, studies have demonstrated the effectiveness of Icx vaccines even in the presence of maternally derived antibodies (Giambrone *et al.*, 2001).

A vectored vaccine utilizes one organism as a carrier (vector) to generate protection against the second organism. Avian vectored vaccines are often bivalent vaccines since the vector is usually a vaccine strain. Vector vaccines consist of two essential components: the vector itself and the foreign sequence integrated into its genome. The integrated sequence facilitates the expression of the protective gene specific to the targeted pathogenic agent during the vector's *in-vivo* replication. Subsequently, the produced protective protein initiates an immune response against the agent, protecting the vaccinated host. The induced immune response includes humoral, cellular as well as mucosal immunity only if the vector replicates in mucosal tissues. Vectored vaccines are particularly useful in differentiating infected from vaccinated animals, especially for the eradication of livestock diseases. They can elicit neutralizing antibodies to a limited number of expressed antigens and this distinct immune response allows for differentiation from that generated through natural infection.

The most common viral vectors used for vaccines in poultry diseases are attenuated fowl pox virus (FPV (Boyle and Hiene, 1993) and HVT (Sondermeijer *et al.*, 1993)). These are large DNA viruses whose genomes can be manipulated easily, and vaccines prepared from the vectors are phenotypically stable and are rarely transmitted horizontally (Bublot *et al.*, 2006, Calnek *et al.*, 1979). Furthermore, they can accommodate the insertion of relatively large foreign sequences that are stably integrated into the genome. However, recombinant FPV vaccines do not revert to virulence but vaccines are not effective in the presence of pre-existing immunity against the vector. Because of the interference of maternally derived antibodies (MDA) with the replication of fowl pox virus, there is an impaired immune response to the transgene (Bublot *et al.*, 2006). Recent focus has shifted towards HVT based vectors as a result of their higher efficacy. NDV serves as an emerging vector for developing vaccines against ND and other poultry viral diseases. Its ability to induce humoral, cellular, and mucosal immunity, along with its stable accommodation of various vaccine candidates such as IBD, IB, Reo, and chicken anaemia virus, establishes NDV as a notable vaccine vector for poultry viral diseases. The present paper reviews the various new generation vaccines available against a plethora of poultry viral diseases.

Infectious bursal disease

Infectious bursal disease (IBD) is caused by a double stranded RNA virus belonging to the family

Birnaviridae and genus Avibirnavirus (Dey *et al.*, 2019). IBDV is one of the most common poultry viral diseases causing high economic losses in the poultry sector by inducing immunodeficiency in chickens and making the birds susceptible to other viral, bacterial, and parasitic infections.

The whole genome of IBDV is divided into segments A and B. The large open reading frame-1 (ORF) of segment A encodes for a 110 kDa polyprotein which is spliced into VP2, VP3, and VP4 viral proteins and ORF 2 encodes for VP5. VP2 is the principal host protective antigen and capsid protein of the virus which induces neutralizing antibodies (Dey *et al.*, 2017). IBDV genotypes are produced based on the amino acid changes in the hypervariable region of VP2 protein. Genome segment B encodes a 98 kDa protein that is VP1 which represents the viral RNA dependent RNA polymerase.

In the early days, control of IBD was achieved through active immunization of progeny with modified live vaccines (MLVs), which induced a protective immune response once the MDA level declined (Hein *et al.*, 2021). Similarly, inactivated vaccines also have played a major role by administering them before the onset of laying to boost the immune response elicited by MLVs. Variant strains of the virus have emerged because of antigenic shift (Snyder *et al.*, 1988) and subsequently, these conventional MLVs and inactivated vaccines have not given proper protection against these strains. Because of the low immunogenicity and reversion of virulence in the conventional vaccines, genetically engineered vaccines have been developed with improved safety (Prandini *et al.*, 2016). Determination of complete sequence of the IBDV genome has accelerated the research toward the development of a reverse genetics vaccine system (Mundt *et al.*, 2003). Adaptation of very virulent IBDV to tissue culture needs site directed exchanging of amino acids and converting them to attenuated IBDV which is then rescued by reverse genetics (Van Loon *et al.*, 2002, Raue *et al.*, 2004). This attenuated mutant strain can protect both variants as well as classical IBDV strains.

Recombinant subunit vaccines based on the expression of VP2 protein alone could potentially enable the development of the DIVA strategy. Various expression systems including *Escherichia coli* (Rong *et al.*, 2007), yeast (Fahey *et al.*, 1991, Dey *et al.*, 2009), fowl pox virus (Heine & Boyle, 1993), baculovirus (Vakharia *et al.*, 1993), Semliki Forest virus (Phenix *et al.*, 2001) and even plant expression systems (Wu *et al.*, 2004) have been employed to express either recombinant VP2 (rVP2) alone or as a polyprotein gene. Experimental vaccination studies employing rVP2 have reported varying degrees of protection ranging from partial to 100%. Notably, three vaccines based on the VP2 subunit have been introduced commercially to the market with VP2 expressed in the

baculovirus system, *E. coli*, and the yeast *Pichia. pastoris*. Like the inactivated vaccines, the limitations of parenteral administration and the need for booster immunizations impede the widespread use of subunit vaccines.

Generation of VLPs is an emerging strategy for enhancing the immunogenicity of IBDV subunit vaccines because they are similar in structure to natural viral capsids and are effective at inducing a humoral response. The baculovirus system is the most frequently used to produce IBDV VLPs but is inefficient because of its high cost and low yield. Comparatively, *E. coli* expression systems are more economical with high production of recombinant proteins on an industrial scale but their immunogenicity is less (Ji *et al.*, 2023). Non-infectious subunits of pathogens, including recombinant proteins, peptides, or sugars exhibit low immunogenicity and require formulation with immune stimulating adjuvants. The immunogenic response elicited by a VLP based vaccine surpassed the recombinant VP2 subunit and IBDV polyprotein expression products (Martinez *et al.*, 2003). Expression of IBDV VP2 in yeast (*Saccharomyces cerevisiae*) resulted in the formation of subviral particles (SVPs) with a diameter of approximately 20 nm (Dey *et al.*, 2009). These SVPs based IBD vaccines provided complete protection against very virulent strains and elicited robust humoral and cell mediated immune responses (Maity *et al.*, 2015).

IBDV Icx vaccine efficacy was experimentally found to be equal to or better than that induced by vaccination with live IBDV vaccines. Complexing of IBDV with specific antibodies resulted in a delayed virus detection of approximately 5 days with a remarkably low level of depletion of bursal and splenic B lymphocytes (Jeurissen *et al.*, 1998). The immune complex will remain bound to the surface of follicular dendritic cells present in the spleen and bursa until the MDA decreases sufficiently to a point at which the infectious IBD strain starts to replicate in the bursa without causing immunosuppression.

Using recombinant live viral vectors such as NDV (Guo *et al.*, 2023, Dey *et al.*, 2017), Fowl pox virus (Eldaghayes *et al.*, 2023), Marek's disease virus (Li *et al.*, 2016) and avian adenovirus (Francois *et al.*, 2004), birds are protected against IBD by incorporating VP2 gene as a bivalent vaccine. The herpes virus of turkey is apathogenic in chickens and has been used as a vector for a long time in vaccines for protection against IBD (Darteil *et al.*, 1995). Bivalent vaccines against both MD and IBD based on HVT vectors expressing VP2 of IBDV were shown to be efficacious and safe (Tsukamoto *et al.*, 2002). They can be inoculated into embryonated eggs as well as day old chickens and are effective in the presence of high titres of maternally derived antibodies (Bublot *et al.*, 2007).

The rHVT-IBD vaccines provided excellent protection against variant IBDV, virulent IBDV and very virulent IBDV (Gelb *et al.*, 2016). The onset of humoral and cell mediated responses was detected from 18 to 22 days post vaccination (Ingrao *et al.*, 2017). Prolonged protection and appreciable antibody titres were observed in layers vaccinated at 1 day of age till 30 weeks of age (Tsukamoto *et al.*, 2002). However, the disadvantage is that administering two different rHVT vaccines to the same bird is not advisable, as the immunity triggered by the virus with faster replication can neutralize the virus with slower replication. To address this issue, trivalent vaccines have been introduced to the market (Romanutti *et al.*, 2020).

Infectious bronchitis

Infectious bronchitis virus (IBV) is an enveloped positive-sense single-stranded RNA virus belonging to the family Coronaviridae and genus Gamma coronavirus. The virus is highly contagious and can cause a significant impact on economic losses to commercial poultry farms by affecting all age groups and types of chickens (Zhao *et al.*, 2023, Priyanka *et al.*, 2019). IBV primarily replicates in the respiratory tract but also in the epithelial cells of the gut, kidney, and oviduct.

There are many genotypes or serotypes of IBV strains that co-exist in many countries because of the rapid variation and low cross protection from the vaccine strain against non-related field strains (Zhang *et al.*, 2021). Currently, IBV strains are classified into 7 genotypes (G to G) and 35 lineages (G-1 to 29, G to G) based on the sequence of full-length S1 subunit of the spike gene. The genome of IBV is approximately 27 to 32 kb in size (Cavanagh, 2007) that encodes RNA polymerase/ transcriptase (1ab), spike (S), envelope (E), membrane (M), several accessory (3a, 3b, 5a, 5b), and nucleocapsid (N) proteins (Rohaim *et al.*, 2020). Among those 4 structural proteins (S, M, E, N), the spike (S) protein is immunogenic and frequently exploited for the development of IBV vaccines (Ravikumar *et al.*, 2022). The spike protein of IBV is post transcriptionally cleaved into 2 subunits, S1 and S2 where S2 is anchored into the viral envelope and is important for membrane fusion (Toro *et al.*, 2014). S1 comprises the head domain of the spike and is responsible for the host receptor binding (Lv *et al.*, 2020, Hong *et al.*, 2023). Nucleotide heterogeneity is predominantly observed in the S1 segment of the gene and is primarily confined within three hypervariable regions (Valastro *et al.*, 2016). Because of high mutation rates, the S1 gene is responsible for serotype specificity which generated numerous novel variants of IBV and contains epitopes for neutralizing antibodies (Moore *et al.*, 1997, Zhao *et al.*, 2017). Recombinant vaccines for IBV typically utilize S1 and S2 subunits of the spike protein to generate subunit vaccines. These immunogens are

typically obtained through purification from cell lines or expression using baculovirus vectors (Moore *et al.*, 1997). Although, few studies have demonstrated that S1 alone may not provide sufficient cross protection against IBV variants (Eldemery *et al.*, 2017), its protective efficacy can be significantly improved by combining with other IBV surface proteins which include M or N proteins. Recombinant baculovirus expressing both S1 and N has been shown to elicit a higher level of antibodies when compared with a single antigen baculoviral vector (Yuan *et al.*, 2018).

An alternative pathway for controlling IBV involves the use of VLPs where their distinct advantage lies in their inactive nature when compared to live IBV vaccines. VLPs are composed of S and M proteins that induce high antibody levels to overcome the disadvantage of low cellular immune response associated with recombinant vaccines by effectively stimulating cellular immunity (Liu *et al.*, 2013). Further-more, a VLP incorporating the S, M, and E proteins of IBV has been shown to prompt elevated levels of neutralizing antibodies when compared to groups vaccinated with dual antigen VLPs.

Viral vectored vaccines provide an alternative approach to live attenuated IBV vaccines wherein vectored vaccine expressing the protective antigen of IBV will not lead to the creation of new recombinant viruses. Several attempts have been made by creating bivalent recombinant vectors for IBV by splicing the S protein gene or its subunits into a variety of viral backbones such as NDV (Abozeid *et al.*, 2019), fowl pox virus (Wang *et al.*, 2009, Chen *et al.*, 2010), herpes virus/duck viral enteritis (Li *et al.*, 2016) and adenovirus (Johnson *et al.*, 2003), amongst which the use of NDV as a vaccine vector for IBV holds significant potential (Abozeid *et al.*, 2019). NDV replicates efficiently in the respiratory tract of chickens inducing mucosal immunity and bringing about strong humoral and cell mediated immune responses, essential for clearance of IBV (Khattar *et al.*, 2015). It has been reported that recombinant NDV expressing only the IBV S2 subunit provided partial protection whereas the IBV S1 subunit also provided partial protection but had improved results with better protection following a booster vaccination. Recently it has been shown that recombinant NDV expressing the S protein of classical IBV M41 strain provided better protection than the S1 or S2 protein of IBV alone (Shirvani *et al.*, 2018).

A single immunization on dayold chicks did not reduce tracheal shedding but reduced the severity of clinical signs. IBV is a highly infectious and fast replicating virus and needs a higher number of neutralizing antibodies and cell mediated immune response for complete clearance of the virus from the respiratory tract. So, booster vaccination at day 14 of age provided better immune protection against the IBV

challenge (Abozeid *et al.*, 2019).

Newcastle disease

Newcastle disease (ND) is caused by the Newcastle disease virus (NDV), belonging to the family Paramyxoviridae and genus Orthoavulavirus. NDV has a single stranded negative sense non-segmented RNA genome. The virus encodes six main structural proteins namely nucleoprotein (NP), phosphoprotein (P), matrix protein (N), fusion protein (F), haemagglutinin neuraminidase (HN), and large (L) proteins (Dey *et al.*, 2014). The genome of NDV is approximately 15 kb (Chellappa *et al.*, 2012). Based on the pathogenicity, NDV can be classified into three pathotypes namely velogenic (highly virulent), mesogenic (intermediate virulent), and lentogenic (low virulent).

Among the factors for determining the virulence of NDV, the fusion protein cleavage site (FPCS) has been characterized as a major determinant of NDV virulence (Peeters *et al.*, 1999). Both mesogenic and lentogenic strains can be used for vaccination but mesogenic strains are used only in older birds above 6 weeks of age. Comparatively, mesogenic strains provide long term protection and have high immunogenicity whereas lentogenic strains require booster vaccination regularly. The frequently used strains of NDV for vaccination are Lasota, F which are lentogenic, and Komarov and R2B are mesogenic in nature. The major viral surface glycoproteins, HN is also immunogenic and elicits neutralizing antibodies in the host during infection. Conventional live attenuated vaccines have been used for the control of NDV worldwide, but it has several drawbacks. For example, these vaccines cause respiratory reactions as well as secondary respiratory infections in combination with other pathogens, offers a short duration of protection (Senne *et al.*, 2004), and interference of anti-NDV MDA with immunization when vaccines are given at young age (Czifra *et al.*, 1998). Inactivated vaccines are usually given as a booster for the birds which are previously vaccinated with live attenuated NDV to induce long lasting humoral antibody response.

Subunit vaccines, the second generation of vaccines, utilize pathogen components like antigenic proteins or fragments with high immunogenicity instead of whole organisms. The subunit vaccines primarily employ F or HN or both proteins that are expressed from bacteria (*E. coli* and *Bacillus subtilis*), animal cell lines (chicken embryo fibroblast and quail fibroblast), insect cell lines (Sf 9 and Sf 21) and plants (corn, potato, and tobacco) (Lye *et al.*, 2023). Lee *et al.*, (2008) showed the immune efficacy of individual F and HN subunit vaccines alone and in combination. The subunit vaccine containing both HN and F proteins, provided better protective immunity than individual subunit vaccines due to their synergistic action, especially after boosters. However,

weak immunogenicity and instability in the mucosal environment need to be addressed to make it a potential commercial vaccine candidate (Lye *et al.*, 2023).

VLPs mimic the morphology and structure of infectious viruses by assembling viral structural proteins and lipids without the viral genome, maintaining their antigenicity. Their non-infectious nature, low potential for recombination, and strong immunogenicity make VLPs a promising alternative to viral vaccines (Tan and Jiang, 2014). Wu *et al.* (2019) demonstrated that chimeric IBD-ND VLPs produced robust humoral immunity comparable to inactivated adjuvant vaccines. These VLPs also induced stronger cell-mediated immunity (both Th1 and Th2) than inactivated vaccines and more effectively reduced viral shedding. The VLP formation with glycoproteins from different NDV strains suggests broader protective efficacy when challenged with different NDV strains. Furthermore, these versatile VLPs can be engineered to express protein sequences from diverse pathogens, indicating extensive applications in vaccine design (Dimitrov *et al.* 2017). However, a drawback of the VLP vaccine is the need for a complicated purification procedure that results in increased cost of the vaccine.

Immune complex vaccine or antigen-antibody complex plays a crucial role in providing a delayed-release mechanism for the vaccine antigens. This mechanism ensures that the antigens are not neutralized by the chick's maternal antibodies until the chick's immune system is mature enough to respond effectively (Jeurissen *et al.* 1998). The mixing of live B1-LaSota NDV and NDV-specific antiserum and injecting through the *in-ovo* route on the 18th day of the embryo provided complete protection against virulent NDV infections in chicks (Kapczynski *et al.*, 2012). This approach induced better local and humoral immunity; however, viral shedding was observed, although at a lower level when compared to the live attenuated vaccines.

As FPCS is a major factor in determining virulence (Panda *et al.*, 2004), a recombinant NDV was generated by changing the multi-basic cleavage site of the F protein to a dibasic sequence (Yadav *et al.*, 2018). This can be done either by site directed mutagenesis or PCR mutagenesis thus generating a mesogenic strain having more immunogenicity with markedly reduced virulence (Liu *et al.*, 2015) as a recombinant viral vector. Subsequently, this emerging vector can be used for bivalent as well as multivalent vaccine preparation against NDV and other poultry viral diseases. NDV can accommodate foreign genes stably as well as induce mucosal, humoral, and cellular immunity. Numerous poultry viral vaccines have been generated by using NDV as a vector which includes vaccine targeting Avian Influenza (AI) virus (Veits *et al.*, 2006), IBDV (Dey *et al.*, 2017), ILTV (Basavarajappa *et al.*,

2014), Reo virus (Saikia *et al.*, 2019), IBV (Shirvani *et al.*, 2018) and chicken infectious anaemia (CIA) (Chellappa *et al.*, 2021).

Herpes virus of turkey

Marek's disease (MD) is a highly contagious disease of poultry caused by a cell associated alpha herpes virus which is commonly known as Marek's disease virus (MDV) (Calnek *et al.*, 1979). MD is an immunosuppressive disease characterized by T-cell lymphomas (Tsukamoto *et al.*, 2002). All three serotypes of MDV have been completely sequenced which are MDV1, MDV2, MDV3 (Herpes virus of turkey-HVT) (Izumiya *et al.*, 2001). Maternally derived antibodies inhibition can be greatly reduced using MDV vaccines, and it can induce long term protective immunity in chickens. HVT has a low vaccine efficacy but is highly safe for chickens and grows notably well in cell culture, whereas MDV1 which has high vaccine efficacy against MD grows slowly in cell culture.

The generation of the first HVT vector vaccine was reported in 1992 incorporating the F gene of NDV (Morgan *et al.*, 1992). The first licensed HVT based bivalent vector vaccine (VAXXITEK® HVT+IBD) incorporating the VP2 gene of IBDV was launched in Brazil during 2006 (Darteil *et al.*, 1995, Bublot, 2015). Each dose of vaccine (0.2ml) to be given subcutaneously contains 3.0 log₁₀ PFU of the live vHVT013-69 recombinant virus. A single dose administered in the hatchery provided long term protection with higher efficacy and protection when compared to a live vaccine. Moreover, the vaccine does not induce bursal lesions and immunosuppression. Usually, the IBD modified live vaccines are administered to chickens either once or twice only when MDA levels have dropped sufficiently and allow the virus to replicate. Similarly, for the immune complex vaccine, though can be given at the hatchery, the IBDV remains non replicative until MDA levels reach a specific threshold (Jeurissen *et al.*, 1998). Due to this immunity gap, the birds remain sensitive to IBDV infection. In contrast, the HVT vectored IBD vaccine initiates replication soon after the hatchery administration which clearly shows the vaccine efficacy even in birds with elevated levels of passive anti-IBDV MDAs. The early immune response facilitated by the replication of the HVT virus and expression of the IBD VP2 gene plays a crucial role in compensating for the reduction of passive MDAs. This ensures that overall antibody levels are constantly above the protective threshold (Bublot, 2015).

As per earlier studies, the F gene of NDV was inserted into the genome of HVT and the resultant bivalent vaccine was produced in the early 1990s. Commercial HVT vectored ND vaccine (Vectormune® ND) elicited durable antibody immunity and provided good protection, but the onset of immunity was delayed compared to the modified live vaccine. Live ND vaccines are needed to

be used in addition to the HVT-ND, to compensate for their slow onset of immunity (Palya *et al.*, 2014, Bublot, 2015, Hu *et al.*, 2022).

Similarly, HVT vectored vaccine is also used as a bivalent vaccine strategy for ILTV as well as AI. HVT-ILT contains either glycoprotein D (gD) and glycoprotein I (gI) (Johnson *et al.*, 2010) or a truncated form of glycoprotein B (Esaki *et al.*, 2013). HVT-AI contains a haemagglutinin (HA) gene from a highly pathogenic AI H5N1 strain (Rauw *et al.*, 2012). These two vaccines also have the disadvantage of delayed onset of immunity similar to that of HVT-ND vaccine (Johnson *et al.*, 2010; Rauw *et al.*, 2012).

Recombinant viral vectors can be modified to accommodate foreign genes from two different pathogens to broaden the scope of protection. For example, a trivalent vaccine has been produced by incorporating IBDV and NDV (VAXXITEK® and INNOVAX®) as well as IBDV and ILTV (Farmune®-HVT-IBD-ILT) into rHVT vectors (Romanutti *et al.*, 2020; Hein *et al.*, 2021).

Advancements in genome editing through CRISPR-Cas 9 have opened new ways for the development of multivalent viral vectored vaccines. Through this novel technology, the avian herpes virus genome could be edited to generate HVT recombinants (Tang *et al.*, 2018). This can be utilized for precise mutagenesis of viral vectors including HVT (Tang *et al.*, 2020), ILTV (Atasoy *et al.*, 2019), and duck enteritis virus (Zou *et al.*, 2017). With CRISPR-Cas 9 technology, the first rHVT triple insert vaccine (rHVT-IBD-ILT-AI) has been developed with three different expression cassettes viz., IBDV-VP2, ILTV gD - gI and AIV H9 HA (Tang *et al.*, 2020). This triple insert vaccine was stable *in vitro* however the stability and efficacy of the vaccine *in vivo* remain to be assessed.

CURRENT TREND OF RECOMBINANT VACCINES IN INDIA

In India, the poultry industry is significant, and the research institutions, government, and private companies are very much interested in investing in technologies and vaccines to improve poultry health and production. Live and attenuated vaccines are widely employed for the prevention of poultry viral diseases such as Newcastle disease, infectious bronchitis, infectious bursal disease, Marek's disease, Reo, and others. Nowadays, most poultry vaccine industries in India have shifted to multivalent vaccine preparations that provide broad-spectrum protection against multiple pathogens in a single vaccination event which simplifies the immunization process for the farming community as well as reduces stress on the birds. For example, vaccines like Polyvax (Indovax Pvt. Ltd.) is an inactivated vaccine that gives protection against NDV, IB, and IBD, Volvac

Ac+ND+IB KV (Boehringer Ingelheim Pvt. Ltd.) is a killed vaccine that gives protection against infectious coryza, NDV and IB. Boehringer Ingelheim India Pvt. Ltd. has launched its recombinant vectored poultry vaccine VAXXITEK® HVT+IBD during 2021 and made it commercially available. As we saw earlier, it is a single-shot vaccine, ensuring lifelong protection against both IBD and MD. The Drugs and Cosmetics Act, The Central Drugs Standard Control Organization (CDSCO) of India has approved some important recombinant vaccines by ensuring the safety, efficacy, and quality of the products imported and commercially released in the country. Imported vectored vaccines that have been approved by CDSCO since 2017 were Innovax-ND (Intervet India Pvt. Ltd.), Vectormune ND (Ceva Polchem Pvt. Ltd.) and Vaxxitek HVT+IBD+ND (Boehringer Ingelheim India Pvt. Ltd.). Innovax ND and Vectormune ND are recombinant HVT vectored viruses with inserted NDV F gene live frozen vaccines and Vaxxitek HVT+IBD+ND is a polyvalent vectored vaccine against MD (serotype 3), IBD VP2 gene and NDV F gene. Immune complex vaccines that have been approved and imported are Novamune and Nextmune which are IBD Icx live frozen vaccines (Ceva Polchem Pvt. Ltd.) for the active immunization of broiler chickens against IBD.

CONCLUSIONS

In the face of growing global demand for poultry meat and products, commercial farms face the challenge of sustaining the essential supply of protein for human consumption. Vaccination stands as the sole line of defense by preventing poultry from major economical viral diseases. In the past few decades, recombinant vaccines have emerged as a promising tool for the control of many poultry viral diseases such as IBD, ND, IBV, MD, and AI. This newer technology has shifted vaccine administration from the field to the hatchery which provides a more controlled environment for vaccination. Moreover, the new generation of vaccines is increasingly incorporating protective immunogens sourced from multiple species to offer simultaneous protection against multiple viral species. Though subunit vaccines and VLPs vaccines are considered a safer alternative to conventional vaccines and have successfully been utilized as novel vaccines against several pathogens, but complex purification procedures and poor stability in field conditions have reduced their commercial applications. Icx vaccine has also been found to be safe and efficacious and can be administered on day 1 of the chick in the hatchery or even *in ovo* at day 18 of incubation. However, the virus can only replicate when the maternal antibody (MDA) level has dropped sufficiently. Vectored vaccines are mainly based on HVT, NDV as well as fowl pox vectors. Viral vectored vaccines are administered in a

single dose as discussed earlier and have the advantages of being bivalent as well as multivalent. Several studies have been conducted for the insertion of numerous protective genes into the HVT vectors to provide simultaneous protection against concurrently circulating viral species which can be commercialized in the future. Newly emerging NDV vectored vaccines have also shown interesting features which when combined with other genes induced clear protective immunity. The future studies on vector vaccines should concentrate more on addressing the existing issues like achieving rapid onset of immunity, providing broad protection without interference by MDAs, ensuring the elicitation of robust mucosal immune response, and developing successful polyvalent vector vaccines without interference by another pathogen. Recombinant vaccine technology can now be enhanced by utilizing consensus-based sequences and adopting approaches that target conserved regions and neutralizing epitopes. These innovations contribute to the development of more effective vaccines against viral pathogens characterized by genetically diverse lineages.

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Marek's disease - Current status and future perspectives

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ABSTRACT

Marek's disease (MD) is a condition caused by a tumor-causing alphaherpesvirus. This virus is known to produce an increase in the number of lymphocytes, particularly T-cell lymphomas, in several organs of the body and the peripheral nerves. The genome consists of linear 160-180 kilobase double-stranded DNA. The earliest publication on MD was authored by Dr. József Marek in 1907. The genetic vulnerability or resistance to Marek's disease has been extensively studied in chickens. The disease is extremely contagious and primarily spreads by airborne transmission. The host's immune system reacts to MDV infection by activating both innate and adaptive immune responses. The occurrence of Marek's disease varies based on the pathotype and the sensitivity of the host. The main pathological abnormalities observed in MD are nerve lesions and visceral lymphomas. Diagnosis in the field relies mostly on the clinical manifestations and postmortem abnormalities. In addition to the aforementioned approaches, virus isolation, identification of numerous viral markers in tissues, genomic detection tests, as well as antibody detection using ELISA, are employed to diagnose MD. The strains utilized for immunization include HVT, SB-1, and CVI988. Immunization against MDV with these strains provides effective protection. Although the vaccination schedule is effective, MD remains a significant danger to the poultry industry due to the emergence of novel pathotypes. Therefore, genetic resistance and stringent biosecurity measures will be crucial supplementary factors to vaccination in managing the disease.

Keywords: Marek's disease, pathotypes, lesions, diagnosis

INTRODUCTION

Marek's disease (MD) is a condition caused by a cancer-causing alphaherpesvirus. It is a frequently seen agent that stimulates the growth of lymphocytes, leading to the development of T-cell lymphomas in different internal organs and peripheral nerves. The disease induces potent immunosuppression and neurological abnormalities, ultimately resulting in mortality and/or health consequences in vulnerable domesticated and wild bird species (Kannaki and Gowthaman, 2020).

THE CLASSIFICATION SYSTEM OF MDV

MDV is classified as a member of Group I (dsDNA) in the order Herpesvirales, belonging to the family Herpesviridae. It falls under the sub-family Alphaherpesvirinae and the genus Mardivirus, specifically the species Gallid alphaherpesvirus 2. In addition to Gallid alphaherpesvirus 2, other significant species in the Mardivirus genus include Gallid herpesvirus 3 (serotype 2) and Meleagrid herpesvirus 1 (serotype 3/Herpesvirus of turkey, HVT) (Worku, 2022). Gallid alphaherpesvirus 2 is responsible for causing oncogenic Marek's disease. Gallid herpesvirus 3 and Meleagrid herpesvirus 1, on the other hand, have been identified as non-oncogenic species. These viruses were recovered from chickens and turkeys that appeared to be in good health. The process of determining the type of serotype 1 Gallid alphaherpesvirus 2 is based on its level of virulence. This virus is then classified into distinct pathotypes, including mild MDV (m MDV), virulent MDV (v MDV), very virulent MDV (vv MDV), and very virulent plus MDV (vv+ MDV) strains (Sun *et al.*, 2017), (Teng *et al.*, 2022).

Genomic Structure

The genomic architecture of alphaherpesviruses follows a standard pattern, consisting of a unique long sequence (UL) and a unique short sequence (US).

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These sequences are surrounded by sets of inverted repeat sequences, namely the terminal repeat long (TRL), internal repeat long (IRL), internal repeat short (IRS), and terminal repeat short (TRS) (Fig 1) (Davidson and Nair, 2004). The MDV genome harbors telomere-like sequences derived from the host cell (Wood *et al.*, 2023). These sequences facilitate the preferential integration of the MDV genome adjacent to the telomeres found in the host cell's DNA, particularly in latently infected cells (Mariappan *et al.*, 2019). The MDV 1 genome consists of 97 individual genes and 114 open reading frames (ORF), some of which are duplicated in repeat regions. The genome also harbors retroviral-like sequences, particularly in serotype I, which are not found in other serotypes.

Transmission Dynamics

The disease is extremely contagious and is transmitted through the air, either by direct contact or by indirect contact with other vulnerable individuals. The infectious material is composed of dander/feather follicle epithelium that contains a fully infectious enveloped virus (Mariappan *et al.*, 2019). The excretion of the contaminated material/dander takes place 2–4 weeks after infection and can persist throughout the bird's whole lifespan. The virus's infectivity, which is linked to feather debris and dander, persists for several months. Upon introduction into a vulnerable chicken population, the virus rapidly disseminates among individual birds, even in the presence of immunization. Darkling beetles (*Alphitobius diaperinus*) also contribute to transmission, albeit to a lesser extent, by indirect means in addition to airborne transfer. There is no clear evidence of MDV being transmitted vertically via the egg. Various variables affect the transmission of Marek's disease within a flock, including the extent of first exposure, the number of vulnerable birds, handling practices, changes in housing, and immunization. Additionally, females are more prone to developing tumors (Nair, 2018).

EPIDEMIOLOGY

MDV isolates are pathotyped by assessing the specific pathology they cause in vaccinated and unvaccinated chickens, as well as their capacity to resist the effects of vaccination. The Avian Diseases and Oncology Laboratory test (ADOL assay) was created by the United States Department of Agriculture (USDA) and is used for pathotyping. Essentially, the ADOL assay evaluates the isolate's capacity to cause lymphoproliferative lesions in vaccinated chickens. The isolates can be categorized into mild MDV (m MDV), virulent MDV (v MDV), very virulent MDV (vv MDV), and very virulent plus MDV (vv+ MDV) strains based on the ADOL assay (Witter and Calnek, 2005). Information regarding Marek's Disease Virus (MDV) outbreaks in various regions of world, along with the virulence characteristics of the field strains, is summarized in Table 1.

The rise in virulence appears to be associated with an increase in oncogenicity, although the molecular basis and mechanisms governing viral virulence are not thoroughly understood (Payne, 2004). As described in table, several MDV isolates of china, exhibited a unique A139 substitution and multiple similar point mutations (E77, Y80, A115, R176, and A217). These amino acid substitutions may potentially impact Meq transactivation activity, potentially contributing to the heightened virulence of Chinese MDV field isolates (Deng *et al.*, 2021). Similarly, meq protein of various MDV isolates of china exhibited the same amino acid mutations at positions 77E, 80Y, and 115A, implicated as a virulent MDV strain

and the commercial MDV vaccine CVI988/Rispens was found inadequate in conferring protection against them (Li *et al.*, 2021).

Likewise, the meq genes of various MDV isolates originating from diverse Asian nations such as Iran, Iraq, and Saudi Arabia, as well as Australian regions including Victoria, Sydney, New South Wales, etc., were found to contain two repetitions of four-proline sequences. This implies a potential for heightened virulence, given the observed negative correlation between the number of four-proline repeats and MDV virulence (Shamblin *et al.*, 2004).

Hosts affected

Marek's disease can affect various avian species, including both domesticated and wild birds. The species listed are Chickens, Domestic Japanese quail, European Common quail, domestic turkey, common bustard, Red Jungle Fowl, sparrow hawk, Ceylon Jungle Fowl, mallard, little owl, mute swan, eagle owl, and domestic goose (Ranjbar and Khordadmehr, 2018).

Genetic predisposition to MD

The severity of the sickness is influenced by the genetic composition of the birds, particularly the alleles that are associated with blood groups. Other species of gallinaceous birds, such as wild turkeys, as well as birds belonging to different orders, are able to withstand infection from MDV due to their distinct genetic resilience. The chicken genotype has been demonstrated to be linked to both susceptibility and resistance to Marek's disease. Due to its inherent genetic vulnerability or resistance to MD infection, genetic methods are being employed to control MD. Therefore, chicken breeders have included MD resistance in the selection processes for breeding stocks (Nair *et al.*, 2020). In addition, genetic resistance may be overcome by exposure to extremely virulent MDVs. Chickens that possess the B21 allele exhibit resistance to MD. This allele is associated with the major histocompatibility complex class I genes, the mechanism of which is still not comprehended. In contrast to the previous findings, Line 61 and Line 71 exhibit expression of the Major histocompatibility complex class I allele (B2). However, Line 61 shows resistance to MD, while Line 71 is sensitive to MD (Burgess *et al.*, 2001).

REPLICATION AND PATHOGENESIS OF VIRUSES

The airborne route is the main pathway via which MDV is transmitted, involving both direct and indirect contact between hens. The shedding of dander containing fully infectious virus can potentially contaminate the environment and infect other vulnerable hosts (Nair, 2018). The development of MD in a host that is vulnerable to the disease can be divided into four clearly defined phases: (a) initial infection causing cell death (cytolytic);

Table 1. Marek's Disease Virus (MDV) pathotypes documented in different regions of world.

S.No.	Continent	Country	Year	Flock type	No. of isolates	Pathotypes	Pathotyping method	Reference
1.	Asia	China	2011-2012	Layers	17	Mixed (vvMDV, vMDV, mMDV)	Meq, gE and gI sequencing	(Yu <i>et al.</i> , 2013)
			2013	Layers	01	vv MDV	Meq gene sequencing	(Gong <i>et al.</i> , 2013)
			2013	Commercial Layers (Hyline)	01(SX1301)	vMDV	(CEF's) and Meq gene sequencing	(Cui <i>et al.</i> , 2016)
			2011-2014	Layer and breeder flocks	06	vv and vv+MDV strains	CEFs and MEQ, ICP4, UL36 genes sequencing	(Lv <i>et al.</i> , 2017)
		Iraq	1995-2020	Genebank samples	50	vv and vv+ strains	Meq gene sequencing	(Deng <i>et al.</i> , 2021)
			2018-2020	Layers (Yellow chickens)	04	vv+ strains	Meq gene sequencing	(Li, <i>et al.</i> , 2021)
		Indonesia	2013	Commercial Layers	09	Likely to be vvMDV	Meq gene sequencing	(Wajid <i>et al.</i> , 2013)
			2014	Commercial Layers	02	Likely to be vvMDV	Meq gene sequencing	(Hartawan and Dharmayan, 2016)
		Saudi A.	2016	Layers	02	vMDV	Meq gene sequencing	(Mohamed <i>et al.</i> , 2016)
		Japan	2016	Broilers	06	vv and vv+ strains	meq, pp38, vIL-8, and gL gene sequencing	(Abd-Ellatieff <i>et al.</i> , 2018)
2.	North A.	Turkey	2017-18	Commercial Layers	09	vv and vv+ strains	Meq gene sequencing	(Yilmaz <i>et al.</i> , 2020)
		Iran	2020-21	Layers	06	vv+	Meq gene sequencing	(Molouki <i>et al.</i> , 2022)
3.	Australia	US	2004	15x7 ab+ chickens	06	1-vv, 05-virulent	ADOL assay (In vivo pathotyping)	(Witter and Calnek, 2005)
			1992	Broiler breeder	01 (Woodlands1)	vv	ADOL assay (In vivo pathotyping)	(De Laney <i>et al.</i> , 1998); (Zerbes <i>et al.</i> , 1994)
		New SW	1994	layer	01 (MPF57)	v	ADOL assay (In vivo pathotyping)	(De Laney <i>et al.</i> , 1998)
			2001	Broilers	01 (MPF132/5)	v	ADOL assay (In vivo pathotyping)	(De Laney <i>et al.</i> , 1998); (Zerbes <i>et al.</i> , 1994)
		Nor. NSW	2002	Broiler breeder	01 (FT158)	vv	ADOL assay (In vivo pathotyping)	(De Laney <i>et al.</i> , 1998); (Zerbes <i>et al.</i> , 1994)
		Victoria	2002	Broiler	01 (02LAR)	vv	ADOL assay (In vivo pathotyping)	(De Laney <i>et al.</i> , 1998); (Zerbes <i>et al.</i> , 1994)
		Sydney	2004	Layer pullets	01 (04CRE)	v	ADOL assay (In vivo pathotyping)	(De Laney <i>et al.</i> , 1998); (Zerbes <i>et al.</i> , 1994)
								(Zerbes <i>et al.</i> , 1994)

(b) a period of hidden infection (latent); (c) a phase of infection causing cell death and immunosuppression; and (d) a phase of transformation (Worku, 2022). After the virus is inhaled, it can be found in the spleen within two days and subsequently discharged into the environment through the feather follicle epithelium as dander after approximately two weeks. The gB glycoprotein expressed by MDV is encoded by the virus and could bind to Heparan sulfate on the surface of the host cell. This glycoprotein is considered a potential cellular receptor molecule for MDV entrance as reported by (Lee *et al.*, 2001).

Following the inhalation of the MD virus, the early cytolitic phase takes place in the B-cells located in the bursa of Fabricius, spleen, and thymus. After the initial cytolitic phase, the MDV enters a latent infection stage where it goes dormant in active T-cells approximately 6-7 days after MDV infection. The virus then spreads throughout the body via the MDV-infected lymphocytes, leading to cell-associated viremia (Silva and Gimeno, 2006). The virus spreads to other organs, including feather follicles. Subsequently, a delayed cytolitic infection takes place in the epithelial cells of the feather follicles, leading to the release of virus particles into the environment through the shedding of feather debris and dander. A small number of T cells that are latently infected transform, ultimately resulting in the development of lymphoma in peripheral nerves and visceral organs (Schermuly *et al.*, 2015). The primary cellular targets that undergo change after MDV infection are the CD4+ T cells. In addition to CD4+ T cells, the virus can alter CD8+ T cells.

CLINICAL SIGNS

The initial manifestation of infection is characterized by the emergence of several non-specific symptoms such as loss of body weight, paleness, decreased food consumption, and diarrhea. Birds affected with Marek's disease have a range of clinical manifestations. Certain hens may perish without exhibiting any discernible clinical symptoms. Neurolymphomatosis is characterized by paralysis in birds, with variable degrees of severity. The paralysis often affects one or more extremities and may progress asymmetrically. Other symptoms include wing drooping, neck twisting (torticollis), crop dilatation, and gasping if the vagal nerve is involved (Singh *et al.*, 2012). Paralyzed birds experience anorexia and perish due to their inability to get food and water. The eyes of birds affected by ocular lymphomatosis, often known as grey eye, experience a loss of their capacity to adjust to changes in light intensity, leading to blindness. In the cutaneous type of muscular dystrophy (MD), broilers are deemed unfit for consumption before slaughter mostly because they have a significant number of skin nodules

or tumors (Das *et al.*, 2018). Acute lymphomatosis does not manifest with paralysis. However, the tumors that develop in the internal organs result in substantial harm to the immune system, leading to impaired functioning and the occurrence of clinical outbreaks of various diseases such as coccidiosis, worm infestations, and Gumboro disease.

Macroscopic Pathology

The main pathological abnormalities observed in MD are nerve lesions and visceral lymphomas. The affected birds exhibit hypertrophy of their peripheral nerves. The brain does not exhibit any noticeable alterations, but, the spinal ganglia may display significant enlargements. The majority of nerves and plexi are affected, with particularly noticeable lesions observed in the sciatic and brachial nerves (Haems *et al.*, 2023). Nearly all visceral organs exhibit macroscopic alterations. The affected nerves exhibit localized to diffuse swelling, grayish/yellowish discoloration, loss of cross striations, and edematous alterations. Lesions can occur on either one side (unilateral) or both sides (bilateral), hence it is necessary to inspect both sides of the nerves in order to identify any alterations. Birds that are exposed to a more aggressive strain of the virus are more likely to develop lymphomas in their internal organs and eventually die. Lymphomas can be seen in various visceral organs including the testis, ovary, lung, heart, mesentery, kidney, liver, spleen, bursa, thymus, adrenal gland, pancreas, proventriculus, gut, iris, skeletal muscle, and skin (Haesendonck *et al.*, 2015), (Ravikumar *et al.*, 2019). Lymphomas that affect internal organs manifest as either localized nodules or widespread enlargements. The cut surface of the nodules has a white or gray coloration, and possesses a hard firmness with a smooth surface. Broiler hens commonly experience condemnation because to cutaneous lymphoma that is typically linked to feather follicles. Cutaneous lymphoma is characterized by the presence of several feather follicles or all follicles, which eventually merge together. Subsequently, in severe instances, these nodules desiccate and transform into scab-like formations with a brownish to blackish crust (Nair *et al.*, 2020).

Microscopic Changes

Peripheral nerves exhibit infiltration of neoplastic cells into the nerves, demyelination and proliferation of Schwann cells, widespread infiltration of tiny lymphocytes, macrophages, and plasma cells, along with inter-fascicular edema, demyelination. Encephalitis of variable severity is observed in the brain. The lesions are distinguished by the inflammation of blood vessels' endothelial cells, the accumulation of lymphocytes and macrophages around the blood vessels, and the subsequent infiltration of big lymphocytes and glial cells throughout the tissue (Gall *et al.*, 2018). Secondary

demyelination is caused by the invasion of lymphoblasts and the presence of large vacuolations in the parenchyma. The lymphomas seen in visceral organs are characterized by their proliferative nature and comprise small to medium-sized lymphocytes, lymphoblasts, reticular cells, plasma cells, and macrophages (Makwana *et al.*, 2018). Macrophages are predominantly observed in indolent neoplasms. The physical appearance of lesions may differ among different visceral organs, yet the cellular composition of tumors remains consistent throughout all organs. Lesions in cutaneous lymphoma primarily exhibit inflammation rather than lymphoma characteristics. The presence of compact clusters of growing lymphocytes, plasma cells, and histiocytes can be observed surrounding the infected feather follicles, indicating the existence of lymphomas. Feather pulp lesions can be used to diagnose conditions before death. These lesions are characterized by lymphomatous lesions that surround feather follicles and contain intranuclear inclusions in the epithelium of the feather follicle (Mariappan *et al.*, 2018).

Morbidity and Mortality

The occurrence of Marek's disease varies based on the pathotype and the sensitivity of the host. While it is true that not all birds displaying symptoms will necessarily die from the disease, some may actually recover (Biggs and Payne, 1967). However, as a general trend, the mortality rate is almost equivalent to the morbidity rate. Before the implementation of vaccination protocols, the death rate varied from 30% to occasionally reaching as high as 60%. Currently, as a result of rigorous immunization protocols, the mortality rate has decreased to less than 5% in the majority of countries (Song *et al.*, 2022). The broiler industry has suffered significant economic losses, primarily due to condemnations rather than mortality, despite the implementation of vaccination measures.

DIAGNOSIS OF MD

Diagnosis in the field is primarily reliant on clinical signs and postmortem observations, such as thickened peripheral nerves, visceral lymphoid tumors, changes in the iris and pupil, and atrophy of the Bursa and thymus. In addition to traditional diagnostic methods, various techniques have been utilized to reach an accurate and definitive diagnosis.

Isolation of a virus

The virus isolation process commonly involves the utilization of white blood cells obtained from blood samples, as well as suspensions derived from visceral lymphoma and spleenocytes. The two most favored cell culture systems for the initial isolation of serotype 1 MDV are Chicken kidney cells (CKC) and Duck embryo fibroblasts (DEF) cultures. On the other hand, serotypes 2 and 3 exhibit good growth in CEF. The initial isolation of MDV was carried out in CKC and DEF cultures (Solomon

et al., 1968), (Mariappan *et al.*, 2019).

Tissue-based indicators of viral presence

The detection of the MDV antigen Meq is a suitable marker for diagnosing MD because it is only produced by MDV-induced tumors and not by avian leukosis or reticulo-endotheliosis induced tumors.

Identification of Viral Antigens

The presence of MD viral antigen in the feather tip can be detected using monoclonal antibodies through Agar gel immunodiffusion (AGID) technique (Scholten *et al.*, 1990). A dot-ELISA technique was developed to detect the presence of the viral oncoprotein "Meq" in tissues of birds infected with Marek's disease virus. This technique was found to have higher specificity compared to the conventional Polymerase Chain Reaction method (Kumar *et al.*, 2016).

Genomic detection assays

The Polymerase Chain Reaction (PCR) and the real-time quantitative PCR (qPCR) techniques enable the differentiation of Vaccine and field isolates (Handberg *et al.*, 2001). A standardized diagnostic method using polymerase chain reaction (PCR) was developed for detecting diseases in the lungs, liver, spleen, kidneys, skin, pancreas, and ovaries. Serotype-specific polymerase chain reactions (PCRs) are crucial for distinguishing between serotype 1 pathogenic herpesviruses and the serotype 3 HVT vaccine strain (Handberg *et al.*, 2001). Nested PCR is capable of identifying the Meq gene of MDV in chickens that are infected. Additionally, it can assist in distinguishing between highly virulent pathotypes and attenuated/vaccine MDV strains (Murata *et al.*, 2007). The quantification of MDV genome copies can be achieved using real-time quantitative PCR (qPCR) as described by (Islam *et al.*, 2004), (Baigent *et al.*, 2005), (Abdul-Careem *et al.*, 2006). The utilization of loop mediated isothermal amplification (LAMP) that specifically targets the meq gene has been demonstrated to be a swift diagnostic technique, particularly when using feather follicles as the sample source (Wei *et al.*, 2012), (Angamuthu *et al.*, 2012).

Antibody detection

The Agar Gel Immunodiffusion (AGID) test is widely employed for the detection of antibodies in chickens that have been infected. The detection of antibodies to MDV was performed using the Enzyme-linked immunosorbent assay (ELISA) technique, as described by Cheng *et al.* (1984) and Zelnik (2004). A more sensitive antigen detecting enzyme-linked immunosorbent assay (ELISA) was developed by Davidson *et al.* (1986) to detect MDV antigens in feather-tips of infected chickens, surpassing the sensitivity of the agar gel immunodiffusion (AGID) method.

MDV VACCINE

All commercially available vaccines against MD are derived from three viral serotypes and recombinant DNA vaccines, which effectively protect chickens. Churchill *et al.* (1969) developed the maiden MD vaccine utilizing an oncogenic HPRS-16 strain of serotype 1 MDV. The HPRS-16 strain was weakened through multiple passages using CKC cultures in order to produce a vaccine. Subsequently, the HPRS-16 strain was substituted with the herpes virus of turkey strain (HVT strain, FC-126) as a highly effective vaccine candidate (Okazaki *et al.*, 1970). The implementation of HVT has effectively reduced the economic losses caused by MD. HVT vaccines are typically administered either as standalone vaccines or in conjunction with the SB-1 strain, a Serotype 2 strain (Morrow and Fehler, 2004). The SB-1 strain refers to naturally non-virulent isolates of serotype 2, as described by (Schat and Calnek, 1978). In general, bivalent vaccines utilize both HVT and SB-1 to harness the combined beneficial effects of serotype 2 and serotype 3 (Witter, 1982).

Vaccines for serotype 1 and 2 are commonly found in the form of cell-associated products (Nair, 2018). The attenuated MDV CVI988 strain is currently the most effective vaccine strain available and is used in multiple countries for chicken rearing purposes (Davidson and Nair, 2004). Marek's disease (MD) vaccines are typically administered in-ovo on the day of embryonation or as a vaccination on the first day after hatching. In addition to the MDV CVI988 strain and the R2/23 strain, there was also a reported attenuated serotype 1 strain called Md11 in the USA during the 1990s. However, it is now considered to provide less protection compared to CVI988 (Witter, 1991). Nevertheless, as more evidence accumulates regarding the ineffectiveness of vaccinations and the emergence of stronger strains (as shown in Figure 5), Marek's disease remains an ongoing threat to the poultry population and results in significant financial losses. A major limitation of MD vaccination is that Marek's disease vaccines are unable to provide complete immunity in the vaccinated host. This means that even though the host has been vaccinated, the virulent virus strains can still replicate inside the host and shed virus particles into the environment, potentially infecting other susceptible hosts. The genetically modified vaccine for Marek's disease, known as vvMDV, which has the Meq gene deleted, has demonstrated successful protection in vaccinated birds by effectively suppressing tumor formation (Lupiani *et al.*, 2004), (Li *et al.*, 2011).

The effectiveness of recombinant fowl poxvirus (rFPV) vaccines, which contain different MDV genes, was demonstrated in studies conducted by (Ross *et al.* 1993), (Nazerian *et al.*, 1996), (Hirai and Sakaguchi, 2001), (Lee *et al.*, 2005). Additionally, Lee *et al.* (2003)

found that rFPV vaccines were effective in chickens that had maternal antibodies against MDV. The Avian myelomonocytic growth factor gene has been integrated into rFPV as a cytokine source, resulting in enhanced vaccine effectiveness against MDV (Djeraba *et al.*, 2002). In addition to these traditional vaccines, the pBAC20 engineered DNA vaccine provided partial defense against infection with the virulent MDV (Tischer *et al.*, 2002). Multiple candidate vaccines have been created previously, however, none demonstrated greater effectiveness than the CV1988 vaccines (Witter and Kreager, 2004).

FUTURE PROSPECTS

Marek's disease is a significant lymphoproliferative/neoplastic illness that affects poultry. It can be efficiently managed through vaccination. Maryland serves as an exemplary system for studying vaccinology, viral oncogenesis, and tumor immunology. Although effective vaccines are currently employed to prevent this disease, the virus persistently undergoes genetic changes, resulting in more aggressive strains that pose a significant health risk to the poultry industry. Therefore, it is imperative to devise viable vaccine strategies that can effectively hinder the emergence of new pathotypes. Hence, it is crucial to consider the inherent genetic resistance of birds to MD during breeding in order to effectively prevent the emergence of new pathotypes. In addition to the standard vaccination protocol, it is crucial to uphold rigorous biosecurity measures on the farm. These measures are essential in effectively managing the disease, working in conjunction with vaccination efforts.

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Prominent infections affecting backyard poultry flocks

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ABSTRACT

Backyard poultry in India is witnessing a considerable improvement in the recent times in terms production, population, and package of practices. Although, backyard chickens are hypothesized for their tolerance to commonly occurring infectious diseases in commercial chickens, significant losses owing to predation, management and, biosecurity issues occur. Newcastle disease, Avian Influenza, infectious bronchitis, fowl pox among viral diseases, mycoplasmosis among bacterial and gastrointestinal helminthiasis and coccidiosis among parasitic disease are commonly encountered. The mortality patterns vary across different breeds or varieties under backyard conditions along with season wise incidence. Heterogeneity variants of Newcastle disease, bronchitis and avian influenza circulate in these flocks which often act as transmissible carriers to other birds without showing symptoms. Therefore, control of backyard diseases is necessary by employing timely vaccination protocols and biosecurity strategies in village chicken production systems.

Keywords: Backyard, Newcastle disease, Gastrointestinal helminthiasis, Coccidiosis

INTRODUCTION

“Rural poultry / Backyard poultry / Smallholder poultry / Family poultry / Scavenging poultry” farming is a traditional system of rearing native or nondescript breeds/ rural poultry varieties and characterized by small units with 5-15 birds per household, primarily reared to meet dietary requirements, instant supplementary cash as per the need and mitigates the risk. Predominantly, maintained in extensive conditions under zero input management strategy (INFPD/FAO/IFAD. 2011). Backyard poultry in India is having paramount importance as it is being used as one of the potential tools to fight malnutrition and poverty (Sambo *et al.*, 2015). India has a rich germplasm of indigenous chicken breeds (19) and their respective crosses for enhancing rural poultry production. As per the 20th livestock census (2019), backyard poultry population in India has witnessed a quantum leap with 45.8% growth rate having 317.07 million and contributing 37% of total poultry in the country. However, due to their low productivity (50-60 eggs/year), they contribute only 20.7% to the total egg production, that fetches higher price than commercial counterpart. Backyard chicken and native birds are generally considered resilient to many diseases despite their frequent exposure to marshy areas and open free-range systems where the incidence of potential pathogens is relatively high and this might be due to the strong innate immune response they elicit. The indigenous breeds have a less history of intensive selection for economic traits as compared to high yielding exotic strains. Indigenous chicken breeds are genetically distinct from other breeds having better immune competence supported by higher complement activity, higher lysozyme and antibody response level Haunshi and Sharma (2002). Various authors reported higher vaccinal immunity titers, better immune gene expression in indigenous breeds than commercial counterparts (Deist *et al.*, 2017; Diest *et al.*, 2018) and Zhang *et al.*, (2018). Recent unpublished findings from our lab indicated the better disease resilience of Aseel chicken than White Leghorn in response to velogenic Newcastle Disease Virus (NDV). However, significant losses in the backyard poultry are common under field conditions and are multifaceted. These are mainly attributed by early chick mortality; lack

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of supplementary feeding in some areas; increased predation risk and; some infectious diseases that affects overall health and production performance of backyard poultry (Fig 1). In addition, lack of facilities for scientific management of the chicks during the initial 4 to 5 weeks in the village conditions and improper veterinary services at the rural corners also contributes for significant losses in backyard poultry. This review mainly aims to address the potential health risks in backyard poultry under field conditions.

DISEASES IN RURAL POULTRY

Backyard poultry in India is comprised of two segments rural and urban backyard rearing systems with average flock size of 12-25 (Samanta *et al.*, 2014) and 12-800 (Yendell *et al.*, 2012) and different managerial

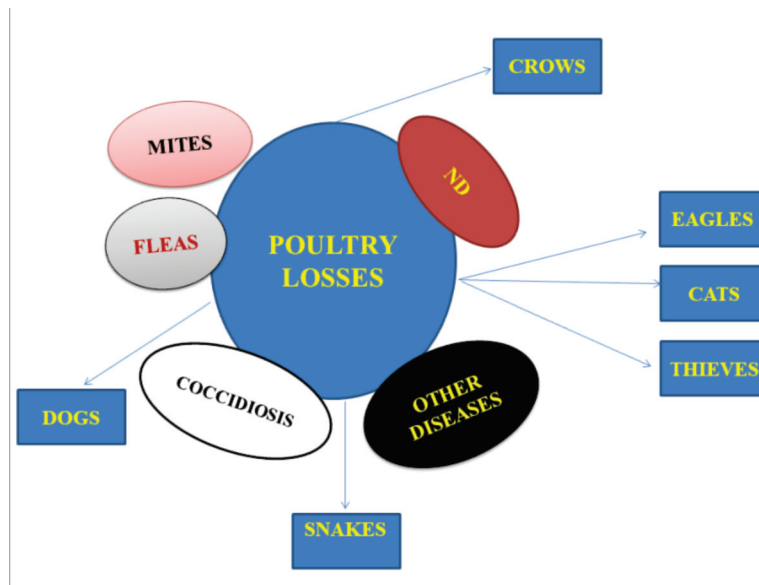


Fig 1. Venn diagram indicating the probable losses and mortality in backyard poultry.

practices. Accordingly, it is expected that infection pressure and disease scenario vary in these birds. Under free range system, there are high chances of backyard poultry to come in contact with migratory birds, waterfowls, wild birds, predators, vermin etc which may increase the risk of infectious disease outbreaks (Conan *et al.*, 2012; Samanta *et al.*, 2018). The global prevalence of disease in backyard poultry flocks was reported as 33%. Among viral diseases, Newcastle disease, infectious bronchitis and avian influenza, prevalence was reported as 30, 71% and 12.5% respectively. Helminths are mostly commonly observed with higher (72%) prevalence (Kannaki *et al.*, 2021).

VIRAL DISEASES

Fowl pox

Fowl pox is an enzootic viral disease characterized by dry and crusty scab like lesions on featherless areas like face, comb, wattles and shank (Skinner and Laidlaw, 2009). The disease is contagious with widespread occurrence across commercial and native chickens with more prevalence in tropical and sub-tropical regions (Giotis and Skinner, 2019) including the vaccinated flocks (Minhas *et al.*, 2023). Presence of pock like lesions on head, face and legs has been reported with a prevalence rate of <1% in Costa Rica backyard flocks and also in USA (Gustafson *et al.*, 2009). It is not uncommon for missing vaccinations for fowl pox in backyard flocks and given the presence of enormous insect vectors this disease pose continuous threat resulting in losses of backyard chickens (Hernandez-Divers *et al.*, 2008). Fowl pox outbreaks in India and Iran were reported by (Das *et al.*, 2013). Jones (2016) also reported the occurrence of fowl pox in backyard flocks of Trinidad.

Newcastle disease

Newcastle disease, a worldwide highly contagious avian viral disease caused by Newcastle disease virus (NDV) of the family Paramyxoviridae in the order Mononegavirales is the most devastating disease of rural chickens (Murphy *et al.*, 1995). The infection was first reported in Newcastle-upon-Tyne in 1926, and the disease is now recognized worldwide (Alexander, 2001). Although its epidemiology is well understood in industrial poultry production systems, little has been published on its behaviour in village poultry populations (Awan *et al.*, 1994). Outbreaks of ND may be devastating, with flock mortality approaching 100% in fully susceptible chickens. In village chicken production, it is single greatest constraint limiting its productivity and development throughout the developing sphere. Velogenic form of NDV is considered endemic in village chickens (Spradbrow, 1993/94) and epidemics result in huge economic losses Martin (1992) by causing even upto 100% mortality (Alders *et al.*, 2010). Apart from poultry, wild and migratory birds, canines, felines and rodents also harbours virus. Feces shed by infected birds act as environmental/inanimate reservoirs for healthy chicken. Heterogeneity within strains of NDV may play an important role in the maintenance and development of infection in village poultry populations. The variant virus forms appear to be sub population complexes that show stability upon many transfers between species. Even several NDV strains circulate in village population enhance the probability of survival of the virus and complicates the disease dynamics. The oral route of infection seems to be more common for the transmission of NDV in free range scavenging village poultry. Seroprevalence of Newcastle disease in backyard

chickens in Somalia, Ethiopia, Mali, Oman Iran and India revealed the prevalence rate of 39.54% (Osman *et al.*, 2021), 6% (Chaka *et al.*, 2012), 58.4% (Molia *et al.*, 2011), 90% (Al Shekaili *et al.*, 2015) and 40% (Saadat *et al.*, 2014) and 9% (Joshi *et al.*, 2021) respectively. Weakness and inappetence are most observed signs in backyard poultry (Gentile *et al.*, 2024). It is probable that NDV in backyard poultry transfers from commercial birds and got adapted in backyard birds that often shows sub-clinical signs with comparatively lesser mortality while shedding the virus (Munir *et al.*, 2012; Singleton *et al.*, 2021). Therefore, it is probable to infer that, backyard chickens sometimes may act as transmission vectors of virulent NDV to commercial poultry flocks (Fagrach *et al.*, 2023).

Avian influenza

Avian influenza is a WOAHC concerned disease affecting all poultry species including avians. Highly pathogenic avian influenza viruses are mainly excreted *via* respiratory tract (Brown *et al.*, 2018; Sturtm-Ramirez *et al.*, 2004), while LPAI are preferentially in feces (Webster *et al.*, 1978). This phenomenon likely poses a significant implication for virus transmission within and between wild and domestic bird populations. Aquatic waterfowl habitats are established source of AI viruses spill over into aberrant hosts like domestic poultry (Hinshaw *et al.*, 1979). AI viruses mainly LPAI ones of wild waterfowls are generally maintained in aquatic habitats through an indirect fecal-oral route. There exists a wide range of environmental fitness phenotypes among strains of same HA subtype (Keeler *et al.*, 2013) but not between HPAI and LPAI viruses and viral factors contributing for this phenomenon are not known and not predictable too (Brown *et al.*, 2018).

Some forms of these viruses, known as highly pathogenic avian influenza (HPAI) viruses, can cause severe illness and a mortality rate approaching 100% (Caussey and Edwards, 2008). However, most strains of these viruses are non-virulent, do not produce clinical signs, or cause only mild respiratory or reproductive disease. These strains are known as low-pathogenic avian influenza (LPAI) viruses and are commonly isolated from many species of wild birds (Alexander, 2000; Swayne and Beck, 2005). Wild migratory birds and waterfowls harbours these viruses in silent form (LPAI) pose a continuous risk to domestic poultry. HPAI viruses, however, are not maintained by wild bird populations but have been isolated occasionally from wild birds during disease outbreaks among domestic poultry.

Large scale epizootics of H5 and H7 subtypes of LPNAI may increase the probability that HPNAI viruses will emerge by mutation. Since 2006, numerous point mutations have expected to have occurred and accumulated in LPAI. This resulted in emergence of drift variants of LPAI in India would have caused severe

outbreaks in the country (Vegad, 2014). Drift variants of low pathogenic avian influenza viruses have become a global challenge, resulting heavy mortality in poultry and are endemic in the Middle East and Asia (Swayne and Halvorson, 2008). With concerted efforts, HPAI virus has effectively brought under control, LPAI is still prevailing by circulating in birds, thereby becoming endemic in India. Since LPNAI has never been reported in India before, it is considered greater hazard than HPNAI in India. LPNAI can transmit via products of poultry and animal origin and pose potentiality for adverse consequences specially when complemented with ND agents, if the disease agents become established in Indian commercial and backyard poultry.

India remained free from H5N1 infection until 2006 (DAFD, 2015). Ever since the inception first outbreak in 2006 in the country, H5N1 emerged as an economically important disease with significant impact on marginal and rural backyard poultry farmers. Country has incurred a total loss of Rs. 2432.08 lakhs from culling of 72.46 live birds, tons of feed and compensation to affected farmers in 168 H5N1 epicenters. Detailed report indicating out breaks in the country is presented in table 1. Majority of the H5NI outbreaks have targeted northern and northeastern states majorly. Within the eastern states, West Bengal, Assam and Tripura have shared the highest burden of outbreaks. These states have a large poultry population with West Bengal having approximately 86 million, Assam with 29 million and Tripura with 3.7 million domestic birds (DADF, 2012 a, b). Recently, Pandit *et al.* (2013) investigated the spatio-temporal clusters and transmissivity of HPAIH5N1 outbreaks in West Bengal. They also investigated the role of wild bird staging areas as risk factors in the epidemiology of HPAI outbreaks in poultry in West Bengal.

The outbreaks have occurred as epidemic waves during 2008-09 and thereafter established as sporadic occurrences. Frequent hitting of NE and eastern regions with H5N1 infections clearly indicating a massive border migration involvement and this must be controlled with stringent security measures. Eastern states share borders with Nepal, Bangladesh, Bhutan and Myanmar and it is increasingly being recognized that HPAI is now a regional cross-border problem with porous borders and illegal movement of poultry and poultry products contributing to the threat of potential endemic circulation within the region. Unlike other countries, India is continuously experiencing a frequent HPAI H5NI strain alone (DADF, 2014). Genome characterization and phylogenetic analysis reports from ICAR-NIHSAD, Bhopal of this strain from 2006-14 outbreaks in the country revealed the origin of three different clades attributing to different areas in different years i.e., clade 2.2 during 2006-2010 (Pattnaik *et al.*, 2006); clade 2.3.2.1-A during 2011 to early

Table 1. Details of HPAI infection forced destruction of backyard poultry in India from 2006-2016.

State (with no. of epicentres)	District	Date of start of event	No. of birds destroyed
Manipur (1)	Imphal East	07/07/07	11
West Bengal (68)	Birbhum	04/01/08	0
	Dakshin Dinajpur	07/01/08	0
	Nadia	15/01/08	113
	Burdwan	15/01/08	0
	Murshidabad	15/01/08	323
	Murshidabad	17/01/08	3181
	Nadia	17/01/08	856
	Bankura	17/01/08	0
	Birbhum	17/01/08	85657
	Bankura	19/01/08	800
Assam (18)	Kamrup	21/11/08	51761
	Kamrup	28/11/08	18292
	Kamrup	30/11/08	183584
	Barpeta	30/11/08	45690
	Nalbari	30/11/08	15964
	Kamrup	05/12/08	37657
	Chirang	05/12/08	5480
	Kamrup	06/12/08	22673
	Dibrugarh	07/12/08	15508
West Bengal	Malda	13/12/08	28695
West Bengal (11)	Murshidabad	19/01/10	2088
Assam (1)	Dhuburi	29/08/11	15409
West Bengal (2)	Nadia	14/09/11	47732

2014 (Nagarajan *et al.*, 2012). Recent H5N1 events in ducks and turkeys in Kerala and ducks in Chandigarh in 2015 are due to clade 2.3.2.1-C (DADF, 2015). In India, around 7.20 lakhs deaths of poultry has been witnessed due to HPAI and LPAI since 2006 (WAHIS, 2024). Recently, avian influenza outbreaks were reported in 47 different states of United States affecting 612 backyard flocks. The positive rate of HPAI and LPAI was 2.53% (115/4538) with H5 subtype as least prevalent in China province (Wang *et al.*, 2018). Infection of backyard poultry with 3.6% seroprevalence rate for LPAI was recorded in New Zealand indicating several potential pathways for transmission of avian influenza to commercial and other poultry species (Zheng *et al.*, 2010). (<https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-commercial-backyard-flocks>).

Infectious bronchitis

Infectious bronchitis is a vertically transmitted and highly contagious disease in chickens including backyard birds with occurrence of three different forms *i.e.*, respiratory, reproductive and nephritic (Cavanagh *et al.*, 2001). 45% prevalence was observed in backyard flocks (Kannaki *et al.*, 2021). In backyard flocks of Oman,

82% prevalence was observed with involvement of 793/B, M41, D274, IS/1494/06 and IS/885/00 genotypes AI-(AI-Shekaili, 2015).

BACTERIAL DISEASES

Mycoplasmosis

Mycoplasma in poultry induces two different infection. Chronic respiratory disease caused by *Mycoplasma gallisepticum* (MG) and synovitis caused by *Mycoplasma synoviae* (MS). It is an economically important disease often characterized by respiratory complexity (Ferguson-Noel *et al.*, 2020) affecting broilers, layers and backyard chickens in addition to turkeys. Higher sero-prevalence of 73% was noticed with MG than MS in backyard poultry flocks located near to commercial premises Derkson *et al.*, 2018 of USA and 31.7% in West Indies (Brown *et al.*, 2018). In Moroccan backyard forms 40.7% and 29.1% seroprevalence of MG and MS was observed (Fagrah *et al.*, 2023). Necropsy based disease diagnosis reported an incidence of 9.8% in USA (Crespo and Cue, 2015). Overall global disease prevalence reported elsewhere in backyard chickens is observed as 60.6% and 76.9% for MG and MS respectively (Kannaki *et al.*, 2021).

PARASITIC DISEASES

Gastro-intestinal helminthiasis

Helminths contributes for majority of health devastation among parasitism in backyard chickens with profound impact (Swayne, 2013; Van et al., 2019). Helminths reduces vaccinal immunity, increasing disease susceptibility, production, and growth (Pleidrup et al., 2014); Rufai and Jato, 2017). The incidence of gastrointestinal parasitism prevails up to 100% and 20% mortality depending upon rearing system (Nnadi and George, 2010). Comparing chicken raised in backyard and free-range systems to those kept in cage production systems, the prevalence of helminth infection was higher (82.6 and 84.8%, respectively) (Shifaw et al., 2021). Overall helminths prevalence of 72% and 92.1% was reported in backyard flocks of India and Ethiopia with 80.2% and 77.8% nematode and cestodes respectively (Katoch et al., 2012); Sarba et al., 2019). Heterakis and Ascariasis of nematodes are the most commonly prevalent helminths in backyard birds (Van et al., 2019). Prevalence of 62.3% of Ascarids and 22.1% of Heterakis was seen in backyard flocks of Karantaka (Javaregowda et al., 2016). Ascaridia was the most prevalent (29.6%) in Jammu regions (Katoch et al., 2012). Of tapeworms, Raillietina and Davainea proglottina have lower occurrence rates. Davainea is the most pathogenic tapeworm while Raillietina induces multiple nodules with 19% prevalence rate (Slimane, 2016); Shifaw et al., 2021). The cestodal occurrence mainly depends on availability of intermediate hosts.

Coccidiosis

Identified by Tyzzer in 1929, coccidiosis continues to affect poultry with severe economic impact, high rates of transmission and wide range of host adaptability. Besides causing production losses, these apicomplexan protozoan compromises host immunological functions and makes highly susceptible to other infectious diseases. Coccidiosis in poultry is often accompanied by Clostridial enteritis and causes enteric illness in different intestinal segments. Coccidiosis is a major parasitic disease that affects the poultry including backyard chickens (Shirley et al., 2005). It is caused by intracellular protozoan parasites belonging to the genus *Eimeria* that are very host-specific (Williams, 1999). Infections caused by the *Eimeriaacervulina*, *Eimeria maxima*, and *Eimeriatenella* are common in intensive poultry systems McDougald et al. (1997), and control of these species, routinely prioritized over other *Eimeria* species. Affected birds tend to loose epithelial lining, shows blood filled caecal pouches with bloody droppings and extreme morbidity (Kahn, 2008). Coccidiosis is reported to have a diverse host range affecting native, layer and broiler chickens and the susceptibility occurs after 3 weeks of age. The overall incidence of coccidial infections was 21.4% with significant correlation with breed, age and sex (Temesigen

et al., 2018) in Ethiopia, 31.8% in Nigeria (Lawal et al., 2016) and 38.36% in India (Singh et al., 2021). Incidence of coccidiosis in backyard range birds of USA was noticed as 9.7% by (Crespo and Cue, 2015).

CONCLUSION

Backyard chickens in India represent an important livelihood for marginal and sub-marginal poultry farmers and is witnessing a progressive in the recent years. Although, considered disease resistant to many infectious diseases, some diseases pose significant risk for the survival of these birds under intensive and extensive farming conditions. Viral diseases such as Newcastle disease, infectious bronchitis, avian influenza and fowl pox; Mycoplasmosis; Parasitic infections such as Gastro-intestinal helminthiasis, coccidiosis are commonly observed diseases with considerable rate of occurrence. Backyard chickens may act as transmissible vehicles to other commercial poultry and integrated health managemental plan with field driven biosecurity practices is often necessary in the current scenario.

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Pseudomonas: An emerging MDR pathogen in hatcheries and poultry farms

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ABSTRACT

Pseudomonas aeruginosa is a serious poultry pathogen and zoonotic microorganism. It is a multi-drug resistance (MDR) opportunistic pathogen, causing acute or chronic infections in hatcheries and poultry farms resulting in significant morbidity and mortality. *P. aeruginosa* displays a vast repertoire of both cell-associated and extracellular virulence factors that contribute to its adaptation to the adverse environment, pathogenesis, successful infection and causing disease.

Keywords: Pseudomonas, Poultry, Pathogenesis, Antimicrobial Resistance

INTRODUCTION

Microorganisms are found ubiquitously in the environment in a vast array of communities, producing thousands of metabolites through complex biochemical reactions (Braga *et al.*, 2016). Poultry farms are also wide open to many bacterial infections resulting in the different clinical sequels that affect the poultry industry economically, affecting the production and/ or transmitting pathogens of zoonotic importance to human beings (Tawakol *et al.*, 2018; Shahat *et al.*, 2019). The microenvironment of hatcheries and poultry farms is ideal for growth of various pathogenic microorganisms and thus serves as a potential source of infection. The hatchery is the leading source for the prevalence of diseases within the poultry industry. The problem starts with contaminated eggs which are incubated. Various bacterial pathogens that contaminate hatcheries have been isolated from dead in shell embryos (Bakheet *et al.*, 2017). Infection may gain entrance to a flock of birds from various point sources, due to errors in management and farm practices.

The microorganisms which can survive for a longer time in the environment are to be considered as the main environment associated disease threat (Kebede, 2010). Such organisms are hardy, resistant to antibiotic treatment and conventional disinfectants and difficult to eradicate. *Pseudomonas* is the one which can be cited as a good example of environment associated infection and may cause a serious problem in hatcheries and poultry farms. *Pseudomonas* spp. is found in many different environmental niches including soil, plants, water, decaying vegetation, humid environments, oil-polluted environments, animals and invertebrates (Bapat *et al.*, 1985; Barnes, 2003; Mohamed, 2004; Joh *et al.*, 2005; Mena and Gerba, 2009; Crone *et al.*, 2020). Extensive metabolic diversity allows *Pseudomonas* to thrive in a wide variety of environments and nutrient sources (LaBauve and Wargo, 2012).

The increasing fear of *P. aeruginosa* is exacerbated by the worldwide problem of antimicrobial resistance (AMR), which has been dubbed a "crisis of the twenty-first century" (Viens and Littmann, 2015). Antibiotic overuse has developed multidrug resistance among bacteria, such as *P. aeruginosa*, posing a severe danger to public health and economies, particularly in low and middle-income countries (LMICs) in Africa and Asia (Islam *et al.*, 2023).

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Members of the Pseudomonaceae family are ubiquitous in natural niches, while birds and smaller mammals have also been described as reservoirs (Behjadi *et al.*, 2021). *P. aeruginosa* is the species type of the genus and has gained prominence as an emerging poultry pathogen with major clinical consequences (Palleroni, 2010). As a versatile opportunistic pathogen, *P. aeruginosa* is capable of causing both acute and chronic infections. It causes serious infections when tissue is damaged.

P. aeruginosa was first isolated from green pus by Gessard in 1882, while the genus *Pseudomonas* was first described by Migula in 1894 (Palleroni, 2010). *P. aeruginosa* is regarded as a normal inhabitant organism of avian spp. but under stressful and immunocompromised conditions, this organism becomes pathogenic (Walker *et al.*, 2002). It is Gram negative, aerobic-facultative anaerobic,

with unipolar motile, encapsulated and nonspore forming bacilli which occurs singly, in pairs or short chains produce water soluble coloured pigments with a specific fruity odour and is known to harbor plasmids that can readily be exchanged (Abd Allah, 1987; Ryan and Ray, 2004; Kebede, 2010).

The word *Pseudomonas* means "false unit", from the Greek *pseudēs* (Greek: ψευδής, false) and (Latin: *monas*, from Greek: μονάς, a single unit). The stem word *mon* was used early in the history of microbiology to refer to microorganisms and germs (Palleroni, 2010). The species name *aeruginosa* is a Latin word meaning verdigris ("copper rust"), referring to the blue-green color of laboratory cultures of the species. This blue-green pigment is a combination of two metabolites of *P. aeruginosa*, pyocyanin (blue) and pyoverdine (green), which impart the blue-green characteristic color of cultures (Palleroni, 2010). Another assertion from 1956 is that *aeruginosa* may be derived from the Greek prefix *ae-* meaning "old or aged", and the suffix *ruginosa* means wrinkled or bumpy (Brown, 1956). The names pyocyanin and pyoverdine are from the Greek, with *pyo-*, meaning "pus" (Tzouchas, 2014) *cyanin*, meaning "blue", and *verdine*, meaning "green". Hence, the term "pyocyanic bacteria" refers specifically to the "blue pus" characteristic of a *P. aeruginosa* infection. Pyoverdine in the absence of pyocyanin is a fluorescent-yellow color.

Pseudomonas aeruginosa genetically and phenotypically is highly flexible and adaptive. It colonizes and infects most of the anatomical and environmental locations. *P. aeruginosa* infections have been detected in all organ systems and other specialized tissues. It is responsible for a wide range of diseases including local infections as well as systemic infections, some of which can be life-threatening (Sadikot *et al.*, 2005).

Entry or transmission route is known as an important factor, which influence the virulence of the bacteria. *P. aeruginosa* infection and transmission may have an endogenous or an exogenous origin (Didelot *et al.*, 2016). Mohamed (2004) and Kebede (2010) who mentioned that infection with *P. aeruginosa* can occur through penetration of the egg shell at early and later stages of embryo life and cause death. Birds most at risk of *P. aeruginosa* infection are those with suppressed immune systems. The infection may occur through skin wounds or contaminated vaccines (John Barnes, 1997). However, even healthy birds can acquire an infection if the bacterium gets into their bloodstream through a break in the epithelium due to trauma, and/or the bacterium is present in sufficiently large numbers.

When excessively high concentrations of particulate matter less than 2.5 µm in diameter are found in poultry houses, it damage the respiratory system and impair

host immunity resulting in secondary infections like *P. aeruginosa* and produce a more intense inflammatory response, resulting in more severe lung injury. Poultry houses are closed breeding environments with high stocking densities and poor ventilation. Since, particulate matter with a complex composition tends to form during poultry respiration, excretion, and feeding processes. Compared with the open-air environment, PM2.5 in poultry houses may also cause more serious injuries to humans (Just *et al.*, 2011; Kearney *et al.*, 2014). Moreover, the opportunistic bacterial pathogen *Pseudomonas aeruginosa* has been consistently detected during compositional analyses of PM2.5 samples from different seasons in poultry houses (Jiang *et al.*, 2018).

Pseudomonas species play a very effective economical role in poultry industry. Various species of *Pseudomonas* viz. *P. cepacia*, *P. fluorescens*, *P. putida*, *P. fragi* and *P. aeruginosa* were reported from hatchery and farm samples (Shahat *et al.*, 2019). But, *P. aeruginosa* is the type genus with the highest importance in avian pathology and most predominant pathogenic *Pseudomonas* species causing mortalities among birds specially chickens (Anzai *et al.*, 2000; Fodor, 2007). Morbidity and mortality rate due to pseudomoniasis ranges between 2 to 100%, but more commonly about 2-12% with greatest losses in very young birds (John Barnes, 1997, Walker *et al.*, 2002). The mortality rate by *P. aeruginosa* is higher than other gram negative pathogens because it has ability to produce several extracellular products and after colonization can cause extensive tissue damage, invade blood and disseminate in body.

Although birds of any age can be infected with the organism, young birds severely stressed or immunodeficient birds usually are the most susceptible. Concurrent infections with viruses, other bacteria and mycoplasmas, enhance susceptibility to *Pseudomonas* infection. In embryos infection with *P. aeruginosa* induces death in the shell, while infection of chicks led to septicemia, respiratory and enteric infections, and high mortality (Stipkovits *et al.*, 1993, Kebede, 2010, Abd El-Ghany, 2021).

The clinical signs include lameness; incoordination; swelling of head, wattles, and hock joint or footpads, diarrhea, and septicaemia. Lesions are consistent with clinical findings and include subcutaneous edema; exudates in affected joints; inflammation of serous membranes; swelling and necrotic foci in liver, spleen, kidney and brain (Barnes, 2003). Septicaemic infection in poultry due to *P. aeruginosa* has been indicated in various reports like Bakheet and Tora (2020).

In chickens, *P. aeruginosa* produces dyspnea and cheesy deposits on the serous surfaces lining the air sacs and peritoneal cavity. In addition, congestion of internal

organs, perihepatitis and pericarditis were reported (Riad, 1994). Histopathologically, pseudomonas infection lesions including mild to severe fibrinosuppurative or fibrinoheterophilic inflammation with presence of rod shaped gram negative bacteria (Boulianne, 2013).

It also induces a significant economic loss to the farm by causing high mortality of newly hatched chickens and mass death of embryos at later stage (Kebede, 2010, Dinev *et al.*, 2013 and Elsayed *et al.*, 2016). The *Pseudomonas* causes infection of yolk sac and degrades the proteins found in the yolk (Walkar *et al.*, 2002 and Dinev *et al.*, 2013). Based on the gross lesions, particularly in the omphalitis and yolk sac infections, the agent may invade the birds through the navel, and spread systemically through the yolk sac. Actually, most reports related to *P. aeruginosa* infection in the chicks considered that the most important source of infection was environment contamination (Devriese *et al.*, 1975, Barnes, 2003, Walker *et al.*, 2002).

P. aeruginosa incidence (24%) from embryonated chicken eggs was recorded and explained that this high incidence in unhatched eggs may be by using uncleaned eggs contaminated with droppings and inadequate disinfection of incubators and hatchers (Bakheet and Torra, 2020). Various studies revealed the presence of organism *P. aeruginosa* in hatchery samples and dead in shell embryos (Al-Sadi *et al.*, 2000; Mohamed, 2004; Kebede, 2010; Elsayed *et al.*, 2016; Bakheet *et al.*, 2017; Shahat *et al.*, 2019; Bakheet and Torra, 2020 and Eraky *et al.*, 2020).

Prevalence of *P. aeruginosa* was recorded by different scientists viz. 6.5 %, 8.75%, 10% and 14% by Badr *et al.* (2016), Farghaly *et al.* (2017), Hasan *et al.* (2017) and Soha *et al.* (2021), respectively. In other reports samples of visceral organs collected from dead birds were found to be positive for *Pseudomonas* spp. (Kurkure *et al.*, 2001; Shukla and Mishra, 2015; Sharma *et al.*, 2017; Hassan *et al.*, 2020 and Soha *et al.*, 2021).

Immunocompromised human patients are also highly susceptible to *P. aeruginosa* infection, and the disease is associated with severe lung damage. The food chain has been found to be an important reservoir of antibiotic-resistant *Pseudomonas* spp. (Heir *et al.*, 2021). Chicken carcass and other poultry retail products play an important role in the transmission of *P. aeruginosa* to humans, after processing. Humans may get infected with *Pseudomonas* spp. through occupational contact with poultry carcasses or in poultry processing plants. *Pseudomonas* spp. are also regarded as important spoilage organisms and can spoil chicken's carcass meat through production of proteolytic, saccharolytic, lipolytic, and biosurfactant changes, especially at the end of shelf life (Morales *et al.*, 2016). The predominance

and persistence of *Pseudomonas* spp. in foods and on food processing surfaces may be related to the ability of these microorganisms to form a biofilm, which enhances their tolerance to harsh adverse conditions, including antimicrobial treatments (Møretør and Langsrud 2017; Quintieri *et al.*, 2019). Treatment of *P. aeruginosa* infection is extremely difficult due to continuous development of antibiotic resistance. The transfer of antibiotic-resistant genes (ARGs) from poultry products to humans creates an additional public health problem.

ISOLATION AND IDENTIFICATION

Among the phenotypic characteristics of *P. aeruginosa*, the characteristic odor (described as flower-like, "grape juice", or "fresh tortilla"), haemolysis and color of the colonies, pigment production, allows for their quick organoleptic identification (Clark *et al.*, 2015). *P. aeruginosa* is a non-fastidious microorganism that does not require special cultivation conditions. The organism is a strict aerobe that grows readily on common bacteriologic media, usually producing a water-soluble green pigment composed of Fluorescein and pyocyanin (Barnes, 2003). It grows well on most of the basic, enriched and non-selective (Nutrient agar, Mueller-Hinton agar, Luria-Bertani, blood agar, etc.) media (Khulod *et al.*, 2012). Isolates of *P. aeruginosa* produce β -zone of hemolysis on blood agar and non-lactose fermenter colonies on MacConkey agar (Bakheet and Tora, 2020). There are some media which are used specifically for the purpose of selective growth of *Pseudomonas* spp. (e.g., Cetrimide agar, *Pseudomonas* Agar Base, King's media, etc.) (Brown and Lowbury, 1965; Kebedy, 2010; Behjadi *et al.*, 2021). On the selective *Pseudomonas* agar base, the organism produces greenish pigment that is characterized by fruity smell. Hook like projections at the periphery of the colonies in *Pseudomonas* agar base media was observed by Kebedy (2010). Khulod *et al.* (2012) used the selective medium Cetrimide agar and incubated at 42°C aerobically which produced greenish-yellow or blue pigment colonies. Serological examination of *P. aeruginosa* revealed that the most predominant serotypes were A, B, D, F, H, K, L, and M. (El-Gohary *et al.*, 2012). While the *Pseudomonas* grows at 37 °C, but can survive at as high as 42 °C temperature (Brown and Lowbury, 1965).

Various biochemical tests are used for identification of *Pseudomonas* spp. (Awaad *et al.*, 1981; El-Shafii, 1992; Barrow and Feltham, 1993; Abd El- Tawab *et al.*, 2014, Shahat *et al.*, 2019). But the biochemical scheme cannot separate various *Pseudomonas* species due to the high resemblance therefore further identification is done by molecular methods to reach to accurate species (Shahat *et al.*, 2019). Many researchers have made attempts to develop PCR for the detection of *P. aeruginosa* (Nikbin *et al.*, 2012; Abd El- Tawab *et al.*, 2014; Farghaly *et al.*,

2017). Identification of *P. aeruginosa* can be achieved by amplification of a substantial produced peptidoglycan associated lipoprotein (*oprL*) gene (De Vos *et al.*, 1997; Abdullahi *et al.*, 2013; Badr *et al.*, 2016). *oprL* gene specific primers encodes a protein in the inner and outer membranes, and is essential for the invasion of epithelial cells and indicate also the pathogenic potential of *Pseudomonas* (Qin *et al.*, 2003; Xu *et al.*, 2004; Hassan, 2013 and Elsayed *et al.*, 2016).

PATHOGENESIS AND VIRULENT ATTRIBUTES

The pathogenesis of *Pseudomonas aeruginosa* infections is multifactorial. *Pseudomonas aeruginosa* attracts the attention as an awful pathogen for its ability to secrete variable arsenal of virulence factors and antibiotic resistance determinants encoded either on plasmids or chromosome which confer remarkable metabolic flexibility and the ability to adapt to multiple conditions, including the host immune response and enables to succeed as an opportunistic pathogen (LaBauve and Wargo, 2012; Morita *et al.*, 2015; Elsayed *et al.*, 2016; Jurado-Martín *et al.*, 2021)

P. aeruginosa displays a massive armory of virulence repertoire (A Wealth of Weaponry) that contribute to its pathogenesis, being controlled by incredibly complex, interconnected regulatory circuits and signalling systems, which give this pathogen great plasticity (Jimenez *et al.*, 2012, Balasubramanian *et al.*, 2013). It has vast number of cell-associated and extracellular virulence attributes which help colonization while others promote bacterial invasion, such as lipopolysaccharide, alkaline protease, elastase, hemolysins, phospholipase "C", rhamnolipids, biofilm, pigments (pyocyanin, pyoverdine), Pilli, and flagella (Herbert, 2003; Gupta *et al.*, 2011, Habeeb *et al.*, 2012; Aljebory, 2018; Shahat *et al.*, 2019; Abd El-Ghany, 2021).

Lipopolysaccharide (LPS) is an important surface structural component to protect the external leaflet and poison host cells and the endotoxicity of the lipid A in LPS enable tissue damage, attachment, and recognition by host receptors (Park *et al.*, 2022). LPS may be related to antibiotic tolerance and biofilm formation (Chambers *et al.*, 2017). LPS exhibits a crucial role in regulating the interaction of bacteria with their host, is the main cause of tissue degeneration and chronic damage.

Out membrane proteins (OMPs) contributes to nutrient exchange, adhesion, and antibiotic resistance (Sabnis *et al.*, 2021). In addition, drug resistance caused by the formation of biofilms is associated with the flagellum, pili, and other adhesins (Ozer *et al.*, 2021). There are six types of secretion systems, including flagella (T6SS-associated), pili (T4SS), and multi-toxin components type 3 secretion system (T3SS), which

function at colonisation of the host, adhesion, swimming, and swarming responding chemotactic signaling. Finally, exopolysaccharides, such as alginate, Psl and Pel, may help facilitate the formation of biofilms, while impairing bacterial clearance (Ozer *et al.*, 2021).

In terms of toxins, T3SS is a complex system and may severely impede host defense via injection of cytotoxins including ExoU, ExoT, ExoS and ExoY, which affect the intracellular environment, especially blocking phagocytosis and bacterial clearance. Exotoxin A (ETA) can inhibit host protein synthesis through ADP ribosylation activity (Karash *et al.*, 2021; Yang *et al.*, 2022). In addition, LasA and LasB elastases, alkaline protease (AprA) LipC lipases, phospholipase C, and esterase A enzymes comprise a large group of lytic enzymes that modulate other virulence factors (Chadha *et al.*, 2021).

P. aeruginosa and other members of the genus *Pseudomonas* are known to produce various pigments, including pyocyanin -a green-blue lipid-soluble phenazone-derivative pigment, with roles in iron metabolism, in maintaining the redox-equilibrium surrounding the bacteria, and in cell-cell communication. Pyocyanin is also toxic to hosts to cause disease severity, damage host tissue, and impair organs' function (Alatraktchi *et al.*, 2020; Chadha *et al.*, 2021). Other pigments are pyoverdine/fluoresceine (a fluorescent green-yellow water-soluble pigment, produced by 70–80% of isolates, which also acts as a siderophore in low-iron conditions), pyorubin (a red-brown water-soluble pigment, produced by 2–3% of isolates, with roles in maintaining the redox-equilibrium), and pyomelanin/alkaptomelanin (a brown-black, water-soluble, and acidic pigment) (Meyer *et al.*, 1996; Hall *et al.*, 2016; Behjadi *et al.*, 2021). It has been indicated that high phosphate concentration in the culture medium induces pigment production in *Pseudomonas* spp. (El-Fouly *et al.*, 2015).

The ability of *P. aeruginosa* to metabolize a variety of nutrients enables them to survive in a variety of habitats by developing biofilms. Within biofilms microorganisms are generally well protected against influence of antibiotics, disinfectants (Hoiby *et al.*, 2010) and host immune system (Jensen *et al.*, 2010). The ability of *P. aeruginosa* to form biofilm renders its ineffective clearance by immune defense systems and antibiotherapy (Rasamiravaka *et al.*, 2015), that is considered as a protective mode that allows bacteria to survive and colonize in hostile environments (Yang *et al.*, 2011).

These factors are regulated by a global regulatory system called quorum sensing (QS). QS allows groups of bacteria to synchronously alter behavior in response to changes in the population density and species composition of the surrounding bacterial community (De Kievit and Iglewski, 2000; O'Loughlin *et al.*, 2013; Bakheet and Tora,

2020). QS is a cell-to-cell communication mechanism among bacteria using small diffusible chemical signaling molecules called autoinducers and depends on the abundance of signalling molecules (autoinducers) in the environment. The signaling molecules accumulate in the surrounding environment with an increase of bacterial density. When the concentration of signaling molecules reaches a minimal threshold, they bind to receptor proteins, thereby activating the expression of genes associated with biofilm formation (Williams, 2007).

P. aeruginosa uses its two major LuxI/R QS systems (the Las and Rhl systems) to orchestrate the synchronous production of virulence factors and to form biofilms (Miller and Bassler, 2001; Waters and Bassler, 2005; Lee and Zhang, 2014).

QS controls virulence and biofilm formation in this pathogen. In *P. aeruginosa*, biofilm formation is linked to chronic infections. Biofilms increase persistence levels against the host immune system, antibiotics and environmental stresses, such as desiccation, oxidative stress or ultraviolet radiation (Flemming and Wingender, 2010). QS-deficient *P. aeruginosa* mutants formed thin and less developed biofilms. Another essential function for QS in *P. aeruginosa* is to regulate the production of multiple virulence factors, such as extracellular proteases (Oh *et al.*, 2017), iron chelators (Kaufmann *et al.*, 2005), efflux pump expression (Dietrich *et al.*, 2006), biofilm development (Sadikot *et al.*, 2005), swarming motility (Blus-Kadosh *et al.*, 2013) and the response to host immunity (Cooley *et al.*, 2008).

The large *P. aeruginosa* genome (~5 Mb to ~7 Mb), which comprises multiple genetic regulatory pathways, is also key to understanding the pathoadaptability of this pathogen, especially with current genomic techniques (Jurado-Martín *et al.*, 2021). Large genome of *P. aeruginosa* is characterized by pronounced genomic plasticity (de Lorenzo, 2015). This genetic repertoire includes a conserved core genome of ~4 Mb, while the remaining genetic material comprises of various sets of rare genes and gene islands (Rumbaugh, 2014). The versatility of this pathogen is largely determined by the latter group of genes.

Outer membrane lipoprotein (OprL) are involved in the integrity of *P. aeruginosa*, implicated in efflux transport systems and affecting cell permeability (Xu *et al.*, 2004). Therefore, it is an important reason for antimicrobial resistance in *P. aeruginosa*.

Flagella mediate swimming and swarming motility of *P. aeruginosa*. It acts as an adhesin and produce cell-to-surface interactions then enable the bacteria to swim and overcome repulsive electrostatic forces that are present between the cell surface and the surface of materials forming film. Flagellum plays an extremely important

role in production and assembly of biofilm formation (O'Toole and Kolter, 1998; Tawakol *et al.*, 2018).

Elastase is a protease that can reduce host defense, immunoregulatory proteins, and impaired epithelia, which is encoded by *lasB* gene (Dulon *et al.*, 2005). *LasI* gene (auto-inducer) is also responsible for the secretion of some virulence tools of *P. aeruginosa* such as elastase and pyocyanin (Bratu *et al.*, 2006; Hassan *et al.*, 2020).

P. aeruginosa uses the virulence factor exotoxin A encoded by the *toxA* gene to inhibit biosynthesis at the level of polypeptide chain elongation factor 2 in the cell, similar to diphtheria toxin, hence eukaryotes can't synthesize protein and necrotize leading to great tissue and organ damage (VanDelden and Iglewski, 1998; Hamood *et al.*, 2004; Eman *et al.*, 2017; Aljebory 2018).

In *P. aeruginosa* four secretion toxins were identified; exoenzyme S, exoenzyme U, exoenzyme T and exoenzyme Y (Garrity-Ryan *et al.*, 2000; Fraylick *et al.*, 2002; Vance *et al.*, 2005). The genes *exoS*, *exoT*, *exoU*, and *exoY* that regulate the action of *P. aeruginosa* type III secretion system capable of injecting toxic effector proteins into the cytosol of host cells and accompanied by elevated mortality rates (Hauser, 2009).

The pathogenicity of *P. aeruginosa* in birds is related to keratitis, keratoconjunctivitis, septicemia, respiratory infections, sinusitis and soared embryonic death rates in hatcheries (Hai-ping, 2009; Hussein *et al.*, 2008).

ANTIMICROBIAL RESISTANCE

The global rise and spread of pathogenic microorganisms invulnerable to existing antibiotics is a worrisome situation. Although some species of bacteria are naturally resistant to antibiotics, the basic principle underneath Antimicrobial Resistance (AMR) development is that bacteria can fix mutations and may acquire extraneous DNA through horizontal gene transfer. These genes do not recognize or respect phylogenetic, ecological or geographical borders. Therefore, resistance resulting in one ecological niche or species may be able to spread with ease to another niche or species (Okeke and Edelman, 2001). The level of resistance continues to gradually increase by the global selective pressure caused by human and veterinary use, misuse and abuse of antibiotics.

Antimicrobials used in poultry production are employed for therapeutic and prophylaxis purposes. Many of these antibiotics employed in poultry production also serve as essential medicines for use in humans in many countries (Dandachi *et al.*, 2018). Due to the misuse of antibiotics in the animal-food production system, the global surge and spread of antibiotic resistance have been developed (Maron *et al.*, 2013). Unfortunately, the rate of

antibiotic resistance of *Pseudomonas* spp. in the food chain also has been increased worldwide (Bonomo *et al.*, 2006).

The microorganisms that belong to the so called ESKAPE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacteriaceae) have capacity to “escape” from common antibacterial treatments (Omoya and Ajayi, 2016). It is included in the “critical” category of the World Health Organisation’s (WHO) priority list of bacterial pathogens for which research and development of new antibiotics are urgently needed (Daikos, *et al.* 2021).

Poultry has been known to be a reservoir of number of antibiotic resistant bacteria that may aggravate the problem of *Pseudomonas* infection. One of the main troublesome characters of *P. aeruginosa* is a minor susceptibility to a lot of types of antimicrobials, making it a very hard pathogen to eliminate and this is because *P. aeruginosa* genome contains the largest known resistance island genes (Balasubramanian *et al.*, 2013 and Khattab *et al.*, 2015). The important reason for antimicrobial resistance is impermeability which belongs to the outer membrane lipoprotein (*oprL* gene) (De Vos *et al.*, 1997). Several mechanisms of resistance to antimicrobial agents have been described in clinical isolates of *P. aeruginosa*.

Resistance is acquired through production of β -lactamases, like extended-spectrum β -lactamases (ESBLs), ESBL genes (*bla*CTX-M, *bla*SHV, *bla*TEM) plasmid-mediated AmpC enzymes, and carbapenem-hydrolyzing β -lactamases (carbapenemases). The ESBL enzymes are capable of hydrolyzing extended-spectrum beta-lactam antibiotics such as penicillins, cephalosporins, carbapenems along with a monobactam (aztreonam) but are inhibited by suicide inhibitors such as clavulanic acid, sulbactam, and tazobactam (Mirsalehian *et al.*, 2010; Odumosu *et al.*, 2015). However, in Gram negative bacteria, lower penetration of the drug through decrease in the expression of outer membrane porin proteins such as OprD of *Pseudomonas aeruginosa* has been reported (Bonomo and Szabo, 2006).

Poultry farms have been using since long the penicillin group of antibiotics for treatment and as growth promoter. Badr *et al.* (2020) and Sharma *et al.* (2017), who recorded 87.5% and 85.71% resistance, respectively to this drug. Monobactams are a subgroup of betalactam antibiotics which work against aerobic Gram negative bacteria like *Pseudomonas* by inhibiting the peptidoglycan synthesis process (Memon, 2021).

Resistance of *Pseudomonas aeruginosa* strains to newer cephalosporins is often associated with stable derepression of synthesis of the chromosomal beta-lactamase (Livermore *et al.*, 1985). The rate of resistance to third generation cephalosporins has increased

among Gram-negative bacteria and Hassan *et al.* (2020) observed high level of resistance (91.2%). Carbapenems, especially meropenem and imipenem, are a crucial group of β -lactams used for the treatment of *Pseudomonas* infections due to their stability to most β -lactamases (Hassan *et al.*, 2020). Recently, the antibiotic resistance profile of *P. aeruginosa* isolates in poultry abattoirs in Nigeria showed a high level of resistance to carbapenems and cephalosporins (Ejikeugwu *et al.*, 2021).

Tetracyclines exhibit the activity against a broad spectrum of pathogenic microorganisms; they are well absorbed, exhibit low toxicity, have minimum side effects and are relatively inexpensive, therefore commonly used in poultry farms (Moellering, 1990; Standiford, 1990; Michalova *et al.*, 2004). Tetracycline resistance can be mediated by the acquisition of mobile genetic elements carrying tetracycline-specific resistance genes, ribosomal protection, mutations within the ribosomal binding site, and/or chromosomal mutations leading to increased expression of intrinsic resistance mechanisms, efflux pumps or chemical modification (Grossman, 2016; Kashefieh *et al.*, 2021).

The most widely used antibiotics in chicken farms other than oxytetracycline are fluoroquinolones like enrofloxacin and ciprofloxacin (Mahanti *et al.*, 2018). Chakraborty *et al.* (2020) reported 80% resistance towards fluoroquinolones.

The systematic and excessive use of antibiotics in the poultry farming sector, without regard for the guidelines for usage, has a negative impact resulting in growth of antibiotic resistance. Antibiotic use generates stress, which can trigger resistance in DNA, resulting in bacterial cell mutations and genetic alterations (Huddleston, 2014).

Apart from this, disinfectants are also used with carelessness which is leading to the adaptation of bacteria and augmenting the spread of disinfectant resistant bacteria. *P. aeruginosa* isolates were found to be resistant to quaternary ammonium compounds (QACs) (Loughlin *et al.*, 2002). It was found that QACs resistance genes were combined decisively with genes coding for resistance to Sulphonamides, Trimethoprim, Chloramphenicol, Aminoglycosides and beta-lactams (Zhao *et al.*, 2012 and Schill *et al.*, 2017).

CONCLUSIONS

Detection of *P. aeruginosa* in poultry and associated environment in various reports is alarming because of the possibilities of transmission of these pathogens to human. *P. aeruginosa*, an important member in the group of non-fermenting Gram-negative bacteria, has received substantial attention in recent years, associated with many different disease manifestations, especially in immunocompromised patients. The emergence of MDR,

XDR, and PDR strains in *P. aeruginosa* is an important public health concern, leading to difficulties in therapeutic choices. Strains of *Pseudomonas aeruginosa* are known to utilize their high levels of intrinsic and acquired resistance mechanisms to counter most antibiotics. In addition, mechanisms like biofilm-mediated resistance and formation of multidrug-tolerant persister cells is responsible for recalcitrance and relapse of infections.

It is usually difficult to clear the poultry farms usually from the *Pseudomonas* organisms since it has high resistant to various antibiotics and may be resistant to conventional disinfectants. Hence, to adopt prevention measures for *Pseudomonas* invasion is an indispensable duty of poultry farmers and the farm management should be committed in implementing all the necessary biosecurity measures. Good and standard hygiene, especially in hatcheries, cleaning and disinfection of equipment and use of sterile techniques in preparing vaccines and injectables will prevent *Pseudomonas* infections. The farm management should take stringent control measures to overcome all the possible sources of *Pseudomonas* infection and judicious use of antibiotics to mitigate the emergence and spread of multidrug-resistant strains.

FUTURE PROSPECTS

To stop indiscriminate use of antibiotics and disinfectants in the poultry farms and the discovery and development of newer antibiotics or alternative therapeutic strategies that present novel avenues against *P. aeruginosa* infections are increasingly demanded in future.

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Current scenario of infectious bronchitis in Indian poultry

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ABSTRACT

Avian infectious bronchitis (IB) is a highly infectious and contagious viral disease of poultry is being one of the foremost causes of economic loss. IB virus (IBV) is categorized under the family Coronaviridae, and genus Coronavirus. IBV has been classified into six main genotypes (GI through GVI) which encompass 32 lineages. In spite of the use of Mass serotype vaccines and rigorous vaccination programs for the control of IBV, it remains as a significant problem in Indian poultry industry. Mass serotype viruses followed by 793b remained the dominant ones in many countries. Recent reports indicate that the incidence of QX IBV variant is increasing reported in many parts of Asia, Africa, Middle East and Europe. The virus has wide range of tissue tropism affecting reproductive, respiratory, urinary, alimentary tract and the muscular system. It causes reduced performance, reduced fertility and hatchability, delayed sexual maturity, increased rate of non-layers, irreparable damage to reproductive tract, poor egg production and quality, nephrosis, increased susceptibility to other infections and costly vaccination programmes. Diagnosis of IB is based on the clinical history, lesions, sero-conversion or rising antibodies titres, IBV antigen detection by antibody-based antigen capture assays, virus isolation and by detection of IBV RNA. Since IBV is distributed worldwide, prevention and control of this disease requires a well coordinated approach, balancing biosecurity / hygienic measures and vaccination.

Keywords: Infectious Bronchitis, Scenario, Lesions, Diagnosis, Vaccination

INTRODUCTION

Poultry rearing in India has taken a quantum jump during the last four decades emerging from a near back-yard practice to a full-fledged Industry with a growth rate of 8-15% per annum (Anjan Goswami, 2023). With a production of nearly 138.38 billion eggs and more than 40 million metric tons of poultry meat India ranks second in world egg production and fifth in poultry meat production DAHD (2023). The incredible achievement has been brought about by the adoption of improved breeding methods, nutrition, management and sophisticated health coverage programmes. Despite these due to selective breeding policy for higher production, intensive rearing and many vaccinations the birds are facing all sorts of stress beyond the limit of their physiological tolerance making them more vulnerable to diseases. Because of this many disease complexes with puzzling clinical manifestations and unknown etiology are emerging in the field causing heavy losses in terms of mortality and production loss in India. Among the prevailing diseases, avian infectious bronchitis (IB) is a highly infectious and contagious viral disease of poultry and it is endemic in India Raja *et al.*, (2020). It is one of the foremost causes of economic loss affecting the performance of both meat-type and egg-laying birds due to reduced performance, reduced fertility and hatchability, delayed sexual maturity, increased rate of non-layers, irreparable damage to reproductive tract, poor egg production and quality, nephrosis, increased susceptibility to other infections and costly vaccination programmes.

HISTORY

Avian infectious bronchitis was first described and reported in North Dakota (USA) by Schalk and Hawn (1931) in baby chicks as highly infectious respiratory disease causing high mortality and named it as Infectious Bronchitis (IB). The viral aetiology of the IB was established by Beach and Schalm (1936). Initially, IB was

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recognized primarily as a disease of young chicks but later it was observed to be common in semi-mature and laying flocks. Van Roekel *et al.* (1951) and Broadfoot and Smith (1954) reported fall in egg production and quality following infection of chickens with IBV. Cumming (1962) reported that IBV was the cause of nephrosis-nephritis syndrome seen in Australia. Subsequently eneterotropic strains of IBV was reported in 1960s, IB strains causing deep muscle myopathy (4/91) was reported in UK during 1990's. After 2000's variant IBVs causing proventriculitis and Ascitis syndrome (QX) have been reported many parts of Asia, Middle East, Africa and Europe (Houta *et al.*, 2021).

ETIOLOGY

IBV is categorized under the family Coronaviridae, and the virus is placed in group-3 of genus Coronavirus that belongs to the order Nidovirales. The virion is round to pleomorphic in shape and possesses an envelope that is approximately 120nm in diameter with a club-shape projection about 20 nm in length (Cavanagh and Gelb Jr, 2008). The name corona virus refers to the corona like appearance formed by the spike-like projections of the spike (S) glycoprotein on the surface of the virus (Davies and Macnaughton, 1979). The nucleic acid of the IBV contains single stranded positive sense RNA, approximately 27.6 kb in length of with a polyA tail (Boursnell *et al.*, 1987). The genome comprised as many as 13 open reading frames (ORFs) in the following order 5'-UTR-1a-1b-S-3a-3b-E-M-4b4c-5a-5b-N-6b-UTR-Poly(A) tail-3. The first 20 kb encode the viral RNA-dependent RNA polymerase and proteases. The remainder of the genome has four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). Among these, the S protein a type I glycoprotein is a determinant of cell tropism (Casais *et al.*, 2003) which is post-translationally cleaved into two subunits; the amino-terminal S1 (approximately 520 amino acid residues) and the carboxyl terminal S2 (approximately 625 amino acid residues) Lai and Cavanagh, (1997). Being located on the surface of the viral membrane plays a crucial role in inducing neutralizing antibodies as well as binding of the virus to host cells and facilitating entry (Promkuntod *et al.*, 2014). IBV has been classified into serotypes, using virus neutralization test or hemagglutination inhibition test; protectotypes, using in vivo cross protection studies and genotypes, using S or S1 gene sequencing and phylogenetic analysis. Based on the full length S1 gene sequence, IBV has been classified into six main genotypes (GI through GVI). These genotypes encompass 32 lineages, among them 27 lineages classified within genotype GI and one lineage each within the remaining genotypes. Apart from that 26 unique IBV variants did not cluster within any of the established lineages (Valastro *et al.*, 2016).

Indian scenario

In 1964, Verma first reported the prevalence of infectious bronchitis virus (IBV) infection of chickens in India (Verma, 1964). In spite of the use of Mass serotype vaccines and rigorous vaccination programs for the control of IBV; IB remains a significant problem in Indian poultry industry. Sukumar and Prabhakar (1993) isolated IBV from an outbreak in Namakkal, Tamil Nadu and confirmed the virus as M41 serotypes by virus neutralization test. Elankumaran *et al.* (1999) identified the presence of M41 and 793/B (4/91) antibodies by HI assay, from samples collected from birds with respiratory system in Namakkal. Until early 2000, India was free of variant form of IBV, the most prevalent form of IBV

being related to the Massachusetts strain (Bhuvaneswari, 2008). However, a novel nephropathogenic variant of IBV distantly related to the 6/82 European strain was reported in India from the outbreaks of visceral gout and nephrosis in commercial broiler chickens in the western parts of India (Bayry *et al.*, 2005). After that the nephropathogenic IBV variants are regularly reported. Subsequently, Gaba *et al.*, (2010) and Sumi *et al.*, (2012) reported existence of 793b variant of IBV by virus isolation and genotyping. The recent reports of Ganapathy *et al.*, (2020) reveal that circulation of QX type of IBV variant in Southern India.

EPIDEMIOLOGY

IBV has been reported in almost all poultry-raising areas of the world. Some distinctly different strains of IBV belonging to a variety of serotypes/genotypes were reported in North America, Europe, Asia, and Australia. Mass serotype viruses followed by 793b remained the dominant ones in many countries with the exception of Australia and New Zealand. Recent reports indicates that the incidence of QX IBV variant is increasing reported in many parts of Asia, Africa, Middle East and Europe (Ren *et al.*, 2020). The reason of most of the field outbreaks are infections with the serologically different strains or variant from the strains used in vaccines. Though domestic chicken has been widely regarded as the exclusive host for IBV, coronaviruses have also been widely reported in turkeys, pigeons, peafowl, partridge and waterfowls (Cavanagh, 2005). Hofstad (1981) stated that all ages are susceptible, but the disease is most severe among young chicks causing mortality. As the age increases, chicken become more resistant to nephropathogenic effects, oviduct lesions and mortality due to infection (Smith *et al.*, 1985). All birds in the flock may become infected, but mortality is variable. Mortality may be as high as 25% or more in chickens less than 6 weeks of age and usually is negligible in chickens greater than 6 weeks (Cavanagh and Gelb Jr, 2008).

TRANSMISSION

The IB virus spreads rapidly among chickens in a flock usually by aerosol inhalation of virus produced by coughing or sneezing chickens. The incubation period, ranges from 18 to 36 hours. Carrier birds become a source of infection for other farms or naive birds. Further transmission from one farm to another farm is facilitated by movement of contaminated people, equipment, and vehicles. The virus can also transmit through the air and travel considerable distances during an active outbreak (Ramakrishnan and Kappala, 2019).

CLINICAL SIGNS

IBV is an acute, highly contagious respiratory

disease occurring in chickens of all ages characterized by respiratory signs, decreased egg production and poor egg quality. The nature and severity of the disease are influenced by the age and immune status of the flock and virulence of the causal strain. The virus has a wide range of tissue tropism affecting reproductive, respiratory, urinary, alimentary tract and the muscular system with diverse clinical symptoms. Kidney damage, enteritis and even pectoral myopathy have also been observed for (Hoerr, 2021).

The characteristic respiratory signs in young chickens are gasping, coughing, sneezing, tracheal rales and nasal discharge (Dhinakar Raj and Jones, 1997). Wet eyes and dyspnea may be seen, and facial swelling may also occur occasionally, particularly with concurrent bacterial infection of the sinuses. In broiler chickens, IBV infection is a major cause of poor feed conversion, reduced growth rate, and condemnation of meat at processing. Broiler chickens infected with one of the nephropathogenic viruses show signs of depression, ruffled feathers, wet droppings, and increased water intake (Li *et al.*, 2022). In laying flocks, decline in egg production and quality are seen in addition to respiratory signs. The severity of the production declines may vary with the period of lay and with the causative virus strain. Deterioration in qualities like watery albumin, mishappened eggs and softshelled eggs are the other manifestation due to permanent damage in oviducts (Mueller Slay *et al.*, 2022).

POSTMORTEM LESIONS

Infected chickens have serous, catarrhal, or caseous exudates in the nasal passages, sinuses and trachea. The air sacs may appear cloudy or contain a yellow caseous exudate (Cavanagh and Gelb Jr, 2008). Nephropathogenic infections produce swollen and pale kidneys with the tubules and ureters often distended with urates (Hoerr, 2021). The relative kidney weight and kidney asymmetry are increased. Despite lack of kidney gross lesions microscopic changes of nephritis may still be present (Winterfield and Albassam, 1984). Fluid yolk material may be found in the abdominal cavity of chickens that are in production, but this is also seen with other diseases that cause a marked drop in egg production. Gross changes in the oviduct caused by early infection may vary from the presence of a continuous patent but underdeveloped structure to a blind sac projecting forward from the cloaca (Ganapathy, 2009). The middle third of the oviduct is most severely affected and may be non-patent and hypoglandular (Gelb *et al.*, 1987).

DIAGNOSIS

Diagnosis of IB is based on the clinical history, lesions, sero-conversion or rising antibodies titres, IBV antigen

detection by antibody-based antigen capture assays, virus isolation and by detection of IBV RNA (Cavanagh and Gelb Jr, 2008). The most common tests used in routine are virus isolation (VI), immunofluorescence assay (IFA), immunoperoxidase assay (IPA) and enzyme linked immunosorbent assay (ELISA). The more traditional methods to determine the serotype of the virus include virus isolation and neutralization tests that are laborious and time consuming and which are now being fast replaced with rapid, specific and confirmatory test like RT-PCR and RFLP (Kwon *et al.*, 1993). The virus is confirmed by isolation in embryonated chicken eggs (ECE) and identified by virus neutralization (VN) tests (Song *et al.*, 1998).

PREVENTION AND CONTROL

Since IBV is distributed worldwide, prevention and control of this disease requires a well coordinated approach, balancing biosecurity / hygienic measures and vaccination. Optimal management practices includes strict isolation, limited controlled site access, separate footwear and equipment for each site/house, and footbaths at the entrance to sites/houses all minimize the risk of introducing the IBV. IBV is easily killed, but applying disinfectants at the correct concentration with a suitable contact time is critical (Bhuiyan, 2021). Hence application of a suitable disinfectant is recommended to reduce infectivity of any remaining virus particles. Generally products containing formaldehyde, chlorine releasing agents, or quaternary ammonium compounds are suitable. The downtime between successive chicken flocks must be maximized (a minimum of 10-15 days is recommended). The control of IBV on multi-age sites is extremely challenging and requires strict control of the movement of personnel and equipment between chicken houses (Legnardi *et al.*, 2020).

Vaccination

Vaccination is key tool of IBV control. Live attenuated vaccines and inactivated vaccines, usually formulated in an oil emulsion adjuvant are commonly used. Live vaccines are used for initial vaccination of breeders and layers. Inactivated oil emulsion vaccines are used primarily at point of lay in breeders and layers. Nevertheless, for inactivated vaccines to be effective birds should have been previously "primed" with a live vaccine (de Wit and Cook, 2019). IBV strains used for live vaccines are frequently attenuated by serial passage in embryonated chicken eggs. Administration of live vaccine can be applied individually by eye drop or intranasal and by mass vaccination (*e.g.*, coarse spray or drinking water). Mass vaccination is usually inexpensive, induce local as well as systemic immunity, could cause some vaccination reaction that is observed for a few days after application (Bande *et al.*, 2017). The commonly used live IB vaccine

strains in India are classical Massachusetts strains such as H120 and Ma5 and variant strains such as 1/96 (IB 4/91). H120 vaccine is a mild vaccine, usually used for first vaccinations, it does not induce a long-lasting immunity. Ma5 vaccine strain is a mild vaccine that can be used as a single component. It can also be included in first vaccination in programs with IB 4/91 vaccines and inactivated vaccines for broad protection against different IBV serotypes (Jordan, 2017). Live vaccines are generally used for initial vaccination of breeders and layers but selection of vaccine strain must be based on the strains prevalent in the area. The occurrence of multiple serotypes of the virus has complicated and increased the cost of disease prevention and warrants the use of local strains in vaccines for their effective control. The inactivated vaccines are used primarily at the point of lay to avoid stress and loss of production. The Massachusetts (Mass or M41) strain is the most popular one as it is the representative of the initial isolates reported from many countries. Inactivated vaccines induce long lasting immunity, show no vaccination reactions, usually cost more than live vaccines, when administered individually allow the combination of different antigens besides Infectious Bronchitis (IB). In order to exploit the potential of the inactivated vaccine the chickens must be properly primed with live vaccines. Highest titres will be obtained when an interval of at least 4-6 weeks is left between the last live vaccine and the inactivated vaccine. Vaccination programs may be simplified by combining inactivated antigens against two or more serotypes or two or more diseases into one vaccine (Bru *et al.*, 2017).

CONCLUSION AND FUTURE PERSPECTIVES

Infectious bronchitis remains one of the major economically important disease of poultry in India and remains as a serious threat to poultry due to emergence of new IBV variants from time to time. IB causes economic losses due to reduced egg production, loss of internal and external quality of eggs, presence of silent layers, infertility, retarded growth, high FCR and increase in the susceptibility to secondary infections. IBV control is achieved by improved biosecurity, hygienic measures and implementation of suitable vaccination programmes. For judicial control of IBV it is advised to use combination of classical mass and variant 4/91 vaccines along with inactivated vaccines at point of lay in layers and breeders. Further regular sero-surveillance in combination with molecular diagnosis will help to identify the vaccine response as well emergence new variants in the field which will help to formulate effective IB control strategies.

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A comprehensive review of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infection in poultry

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ABSTRACT

Avian mycoplasmosis is mainly caused by the pathogenic *Mycoplasmas* *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). Both these pathogens are included in the World Organization for Animal Health listed diseases. Infection is reported in many countries globally and in most of the states in India. Both these pathogens are highly economically important as they can cause huge economic losses in broilers, breeders, and commercial layers due to decreased egg production, weight gain, hatchability, increase in embryo mortality, carcass condemnation, cost of prophylaxis, and treatment cost. Though MG infections mainly affect the respiratory tract and cause chronic respiratory disease (CRD), rarely it causes keratoconjunctivitis, salpingitis, arthritis, and fatal encephalopathy. MS is mainly associated with respiratory tract infection and synovitis in chicken. It can cause peritonitis, arthropathy, and eggshell apex abnormalities. The infection is transmitted both horizontally and vertically. Prevention and control measures for avian mycoplasmosis are based on biosecurity, treatment, and vaccination. Live attenuated, inactivated bacterin and recombinant live virus vectored vaccines are commercially available against these infections. This review summarizes the research studies carried out in India and the world on epidemiology, pathology, and prevention of the disease caused by MG and MS.

Keywords: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, poultry, epidemiology, pathogenesis, pathology, diagnosis, prevention and control

INTRODUCTION

Both the MG and MS are economically significant pathogens of poultry reported worldwide. They are members of the Class *Mollicutes*, Order *Mycoplasmatales*, and Genus *Mycoplasma*. The first described Mycoplasmal disease in birds was infectious pneumo-enteritis in turkeys in England in 1905 and it was later on renamed as infectious sinusitis of turkey. Many species of *Mycoplasma* have been isolated from chicken including MG, MS, *M. meleagridis*, *M. iowae*, and *M. imitans*. Among these, MG and MS are highly pathogenic and very important in poultry worldwide. Both MG and MS were reported from other birds including pigeons, Japanese quail, pheasants and budgerigars. MG and MS infection in chicken and turkey (Levisohn and Kleven, 2000; Kleven, and Ferguson Noel, 2008). Both these diseases are considered to be an important problem in broilers, breeders and commercial layers. Economic losses in the poultry industry caused by these pathogens are significant. In uncomplicated infections in chickens, the morbidity rate is usually high and the mortality rate is low, and a 10- 20% drop in egg production is common during outbreaks. More severe clinical signs occur when the birds are infected concurrently with other pathogens, such as Newcastle disease virus, infectious bronchitis virus, or *Escherichia coli* (Spickler and Anna Rovid, 2018).

MG causes chronic respiratory disease (CRD) in poultry and infectious sinusitis in turkeys. Though MG infections mainly affect the respiratory tract, they may occasionally cause keratoconjunctivitis, salpingitis, arthritis, and fatal encephalopathy (Much *et al.*, 2002). Clinical signs of MG infections in these avian species include rales, coughing, nasal discharge with sinusitis, and severe air sac lesions. Consequences of CRD include mortality, carcass condemnation, reduction in egg production, hatchability, feed efficiency, and

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weight gain (Mohammed *et al.*, 1987; Stipkovits and Kempf, 1996).

MS is another pathogenic *Mycoplasma* associated with respiratory tract infection and synovitis in chicken. It can cause respiratory disease, synovitis, peritonitis, eggshell apex abnormalities, or a subclinical infection and it is considered an important problem in broilers, breeders, and commercial layers. Arthropathic strains can cause a huge loss to layer production due to growth retardation and culling of lame birds. In breeder flocks, loss due to reduction in egg production, reduction in hatchability, and increased

mortality in the offspring has been reported (Stipkovits & Kempf, 1996). In broilers, subclinical respiratory tract infections can lead to the condemnation of carcasses due to air sacculitis. The infection can lead to a reduction in weight gain of up to 20-30%, a 10-20% decrease in food conversion efficiency, and a 5-10% increase in mortality rate (Kleven, 1990). There are pieces of evidence for an increase in the incidence of MS as compared to MG with the emergence of arthropathic and amyloidogenic strains and salpingotropic strains that induce eggshell apex abnormalities (Landman, 2014; Sui *et al.*, 2022).

HISTORY

Mycoplasmosis was first described in turkeys and termed infectious pneumoenteritis by Dodd in 1905 in England (Dodd, 1905). It was later named infectious sinusitis of turkey in 1938 (Dickinson and Hinshaw, 1938). In 1935, Nelson identified cocobacilliformis organisms associated with coryza and in 1943, Delphane and Stuart (1943) named this syndrome as chronic respiratory disease and isolated and cultivated organisms from chickens with CRD and turkeys with infectious sinusitis. Markham and Wong (1952) first reported that the CRD in chicken and infectious sinusitis in turkey were caused by pleuropneumonia-like organisms (PPLO). MS was first reported in 1954 by Olson *et al.* (1954) as an unidentified agent causing synovitis. Later Chalquest and Fabricant (1960) isolated a *Mycoplasma* that was distinct from MG from birds with infectious synovitis and it was named MS (Olson *et al.*, 1964). MS was confirmed as a distinct *Mycoplasma* species by Jordan *et al.* (1981). Kleven *et al.* (1972) first reported that besides causing joint lesions, MS strains can also induce airsacculitis. Feberwee *et al.* (2009) observed that MS induces eggshell apex abnormalities.

Indian Scenario

In India, Chandiramani and Murthy first reported the occurrence of CRD by plate agglutination test in some poultry farms from Uttar Pradesh in 1958 (Jain *et al.*, 1971). Rao (1958) isolated cocobacilliform bodies from a coryza outbreak. Pathak and Singh (1959) isolated *Mycoplasma* organisms from various poultry farms in Uttar Pradesh and West Bengal. CRD was reported, causative *Mycoplasma* was isolated and seroprevalence was studied in different states of the country. Also the outbreaks were reported in and *Mycoplasma* was isolated from turkeys, and wild birds including doves, sparrows, peafowl parrots and pigeons (Jain *et al.*, 1971a; Tripathy *et al.*, 1972; Mall *et al.*, 1975). The MG antigen could be detected in chicken vaginal smears by FAT and its pathology in reproductive and respiratory tracts were studied (Sahota *et al.*, 1977; Rathore *et al.*, 1978; 1979). Pathology of MG co-infection with *E. coli* and the effect of route of inoculation on the pathology of MG in day old chicks were studied (Batra and Singh, 1980; Pruthi *et al.*,

1985). Experimental intra tracheal infection of immune deficient birds recorded a higher gross and microscopic lesion scores and lower antibody response in immune-deficient chickens determined by HI test. (Tiwary and Goel, 1986). The serological response of chickens to MG infection was studied (Chhabra and Goel, 1982; Verma *et al.*, 1988).

TRANSMISSION AND RISK FACTORS

Transmission of both these *Mycoplasmas* can occur by horizontal as well as vertical route (Carnaghan, 1961; Lin and Kleven, 1982). Horizontal transmission occurs by direct contact with infected birds, inhalation of aerosols, through contaminated hatchery, or indirectly through fomites. Both the upper respiratory tract and / or conjunctiva can be the portal of entry of the organism in the aerosols and droplets (Ley and Yoder, 2008). As the mycoplasmas do not survive for more than a few days outside host, carrier birds are important in maintaining this pathogen in farms and an increase in population density increases the rate of horizontal spread. Backyard poultry, wild birds and multiple-aged flocks are important reservoirs for MG (Mohammed *et al.*, 1986; McBride *et al.*, 1991; Sawicka *et al.*, 2020). Even chicken vaccinated in-ovo with live vaccine showed horizontal transmission to unvaccinated birds (Elliott *et al.*, 2019). Both these *Mycoplasmas* have a predilection to the oviduct (Feberwee *et al.*, 2009; Nunoya *et al.*, 1997) and shed the pathogen in the oviduct which helps in vertical transmission. The rate of transmission is reported to be highest during the acute phase of the disease when the level of MG in the respiratory tract is at its peak (Lin and Kleven, 1982). Vertical transmission is the most important transmission route for MS in layers (Ter Veen, 2020) as compared to horizontal transmission. The MG vaccine strain ts-11 is found to be capable of vertical transmission (El Gazzar *et al.*, 2011; Armour *et al.*, 2015). Vertical transmission of MS causes severe economic losses due to embryo death, decrease in hatch rate and post-hatch mortality and also hatchery spread to day-old healthy chicks.

EPIDEMIOLOGY

In *Mycoplasma* infection great variation occurs among MG and MS strains in virulence and tissue tropism, leading to various forms of diseases. Genetic conditions, age, immune status of the host and concurrent infections with IBV, NDV, influenza A, *E. coli*, etc. act synergistically and modify the spread of MS infection (Stipkovits and Kempf, 1996). MG infection is endemic in many large multiple-age commercial egg production units and backyard or free-range poultry flocks which are sub clinically infected and act as sources of infection for commercial flocks (McBride *et al.*, 1991). The occurrence

of disease conditions due to *Mycoplasma* infection varies depending on factors like geographic region, type of bird, age of bird, flock size, time of year, environment and co-occurrence of other respiratory diseases (Yunus *et al.*, 2009; Jalil and Islam, 2010; Seifi and Shirzad, 2012; Bahatti *et al.*, 2013; Rajkumar *et al.*, 2017). There are reports of higher incidence of CRD in summer (Younus *et al.*, 2009; Rajkumar *et al.*, 2017), whereas few researchers have reported higher incidences in winter (Razia *et al.*, 2012; Seifi and Shirzad, 2012). Balasubramaniam and Dorairajan (2009) observed that the season did not influence the incidence of CRD. A higher incidence of CRD was reported in broilers as compared to layers and breeders (Rajkumar *et al.*, 2017), whereas higher seroprevalence in breeder as compared to that of layers and broilers (Shoaib *et al.*, 2019) and a higher prevalence in layers (Shiferaw *et al.*, 2022) were also reported. Higher Prevalence is reported in older birds (Islam *et al.*, 2015; Shiferaw *et al.*, 2022). Chu *et al.* (2014) had detected the presence of MS and MG along with other pathogens in poultry red mites by PCR and suggested that the poultry red mite may play a role as a vector in spreading these diseases in farms.

Prevalence

Since the first report of Mycoplasmosis in turkeys in 1905 in England, the disease prevalence has been reported in countries of the different continents including South and North American continents, Australia, Europe, and Asia (Ferguson Noel, 2012). Infections with MG and MS are serious problems in commercial flocks throughout the world (Levisohn and Kleven, 2000; Kleven, and Ferguson Noel, 2008). Complicated CRD is common in Asian countries. In the last few years, it seems that MS had taken upper hand as compared to MG in commercial poultry (Landman, 2014). Details of the prevalence/seroprevalence studies of MG and MS carried out worldwide are elaborated in Table 1. Infection with MG and MS have been confirmed in several states of India and are presented in Table 2.

PATHOGENESIS

Generally, the upper respiratory tract and conjunctiva are the portals of entry for MG infection. It is primarily a surface parasite of the respiratory tract and conjunctiva, but it also spreads to other organs where acute and chronic diseases occur at multiple sites. Neuraminidase, peroxidase, hemolysins and exotoxins are the important virulent factors that help in causing cell damage (Razin and Jacob, 1992). Colonization of the chicken respiratory tract by MG is mediated by several cytoadhesin-related molecules including MGC1, MGC2, PvpA, GapA, CrmA, HatA, CrmA CrmB, MalF, and vlnA. (Yogev *et al.*, 1994; Papazisi *et al.*, 2002; Indikova *et al.*, 2013; Tseng *et al.*, 2013). Mycoplasmas attach to the plasma

membrane of epithelial cells of the respiratory tract (nasal cavity, trachea, lung and air sacs) by a terminal bleb (Stipkovits and Kempf, 1996). MG derived extracellular vesicles which carry virulence associated proteins have significant impact on immune function and macrophage apoptosis (Wang *et al.*, 2023). A cell surface protein of MG, MG1142 displays homology to the osmotically induced (OsmC) family of proteins and binds strongly to heparin in the extra cellular matrix (Jenkins *et al.*, 2007). Also studies shows that the extracellular proteins such as fibronectin, collagen type IV, heparin and plasminogen play a supportive role in the adhesion or cell invasion of MG (Furnkranz *et al.*, 2013). MG infection causes a significant transient upregulation of immune related genes which leads to the production of cytokines and chemokines in the tracheal epithelial cells that contributes to the infiltration of inflammatory cells into the tracheal mucosa. Increased expression of numerous cell surface receptors, including TLR4 and TLR15 (Beaudet *et al.*, 2017) and proinflammatory chemokines and cytokines, including lymphotactin, CXCL13, CXCL14, RANTES, interleukin-6 (IL-6), and IL-1, and a decreased expression in CCL20, IL-8, and IL-12p40 was reported (Mohammed *et al.*, 2007). Mycoplasmas remain in close apposition to epithelial cells and collagen fibers of the lamina propria which leads to deciliation and degeneration of cells and desquamation of the epithelium (Jordan, 1981). The pathogen initiates an inflammatory and immune response with infiltration of monocytes, lymphocytes and formation of lymphofollicular aggregations with germinal centres in the sub epithelial tissue (Ley and Yoder, 2008).

Studies in the last decade show that MS has a more pronounced respiratory tropism than the articular tropism in poultry. The pathogenicity of MS involves attachment and colonization of the upper respiratory tract and several factors are associated with systemic invasion and lesion production. The MS isolates vary in their ability to produce pathogenic effects in the host and can cause in-apparent infections, air sac lesions and synovitis. (Lockaby *et al.*, 1998; and Morrow *et al.*, 1997). MS has virulent proteins viz. ObgE (a GTP binding protein) involved in transmission, OppF in humoral immune response and GAPDH, the main factor in pathogenesis (Klose *et al.*, 2022).

Both MG and MS have cell invasiveness (Vogl *et al.*, 2008; Buim *et al.*, 2011) which provides them the protection from the immune system and nutritional benefits, and reduces the efficacy of antibiotics and plays an important role in systemic spreading (Much, *et al.*, 2002).

Clinical expression of MG and MS infections varied greatly between different isolates (Truscott *et al.*, 1974; Jordan, 1975; Yoder, 1986; Lockaby *et al.*, 1999; Catania

Table 1. Details of the prevalence/incidence studies of MG and MS carried out worldwide.

Country	Sample type and Number	Bird type	Test	Prevalence (%)		Prevalence/incidence pattern	References
				MG	MS		
Algeria	505 (serum)	Broiler, layer	SPAT	69.9	66.3	Seroprevalence of MG higher during winter season (61.48%) than summer (47.74%), whereas MS infection is more dominant in summer (91.25%) than winter (46.69%)	Heleili <i>et al.</i> , (2012)
Australia	559 (serum)	Layer	ELISA	ND	69	Higher seroprevalence in young birds as compared to adult Positive association between serological status for MS and egg quality parameters like translucency, shell breaking strength, % shell reflectivity and shell deformation	Gole <i>et al.</i> , (2012)
Bangladesh	505 (serum)	Chicken	SPAT	73	ND	Higher seroprevalence in winter season. Highest prevalence (78%) in birds of 65 weeks' age and lowest (71%) in 6-25 weeks' age birds	Raquib <i>et al.</i> , (2022)
	575 (serum)	Chicken	SPAT	55.13	ND	The highest prevalence of MG was in winter (61.6%) followed by autumn (56.9%), rainy (55.0%) and summer (49.6%). Higher in young birds (71.7%) compared to adult (50.4%)	Hossain <i>et al.</i> , (2007)
	382 (serum)	Layer	SPAT	58.90	ND	Highest prevalence found in winter (62.44 %) followed by summer season (53.10%)	Sarkar <i>et al.</i> , (2005)
Belgium	5220 (serum)	Layer	ELIS	0.9	ND	-	Michiels <i>et al.</i> , (2016)
	1224 (swabs)	Broiler	RT-PCR	2.7	12.9	-	
Brazil	210 (serum)	Broiler, backyard chicken, breeder	SPAT HI	4.3 0	7.1 3.8	-	Silva <i>et al.</i> , (2015)
	300 (serum)	backyard chicken	ELISA	53.33	ND	-	Barros <i>et al.</i> , (2014)
China	1,250 (tissues)	Chicken	PCR	75.04	ND	-	Wei <i>et al.</i> , (2023)
	324 (leg samples)	Chicken	RT-qPCR	ND	66.53	-	Wei <i>et al.</i> , (2023a)
	258 (pooled swabs from 258 flocks)	Broiler Layer	PCR	ND	50	Higher incidence of MS infections was in layers (74.1%) than in broilers (20%)	Sui <i>et al.</i> , (2022)
	801 (serum)	Poultry	ELISA	43.1	46.1	-	Hong <i>et al.</i> , (2018)
	44,395 (serum)	Layer	ELISA	ND	41.2	-	Xue <i>et al.</i> , (2017)
Egypt	332 (tissues swabs), Turkey Serum (68)	Broiler, Layer, Turkey	Isolation SPAT	62.0 100	ND	The highest incidence of MG was detected in turkey (83%) followed by broiler and layer chicken. The highest prevalence of MG in chickens and turkey was during the winter and autumn seasons	Marouf <i>et al.</i> , (2022)
	495 (serum)	Chicken	ELISA	47.1	ND	-	Habte <i>et al.</i> , (2022)
	1000 (serum)	Broiler, Layer, Native	SPAT	10.9	13.2	MS infection was more prevalent in older birds and no consistent relationship between MG infection and age	El-Ashram <i>et al.</i> , (2021)
	279 (tracheal swab)	Broiler, Breeder, Layer	PCR	19.7	ND	The MG prevalence was highest in layer (33.3%)	Osman <i>et al.</i> , (2009)

Ethiopia	368 blood	Layer	ELISA	70.65	ND	A higher prevalence (81.76) noticed in adult and zero prevalence in young birds	Shiferaw <i>et al.</i> , (2022)
Iran	100 (swab)	Broiler, Layer	PCR	1.00	72.00	-	Ghadimipour <i>et al.</i> , (2018)
	600 (swabs)	Turkey	PCR	16.6–48.4	ND	Prevalence was higher in backyard (48.38%) than commercial farms (16.66%)	Rasoulinezhad <i>et al.</i> , (2017)
	21 (swabs, tissues)	Broiler	PCR	ND	38.1	-	Ghaniei <i>et al.</i> , (2016)
	2000 (serum)	Layer	SPAT	6.25	18.55	-	Haghighi-Khoshkhoo, <i>et al.</i> , (2011)
Italy	400 (swab)	Layer	Real time PCR	12.5	23.25	The prevalence was 28.6% (commercial) and 40% (rural) for MG, and 42.8% (commercial) and 44% (rural) for MS	Galluzzo <i>et al.</i> , (2022)
Jordan	350 (swab)	Broiler	PCR	6.6	8.9	-	Roussan <i>et al.</i> , (2015)
Libya	103 (serum)	Layer	ELISA (MG & MS)	28.0	-	-	Kammon <i>et al.</i> , (2017)
	1500 (serum)	Broiler	SPAT	3.4	6.4	-	Elgnay <i>et al.</i> , (2013)
Morocco	712 (serum)	Layer	SPAT	58	74.8	-	Fagrach <i>et al.</i> , (2023)
	11 (serum, swabs)	Layer	SPAT	ND	55-100	The Prevalence found increased with the age SPAT (8th week: 55%, 16th week: 91%, 32th and 56th week: 100%), PCR (8th week: 36%, 16th week: 64%, 32th week: 82%, 56th week: 100%)	Nassik <i>et al.</i> , (2014)
			ELISA		100		
Mozambique	459 (serum)	Backyard	Real-time PCR	48.8	36.0-100	-	Messa Junior <i>et al.</i> , (2017)
			ELISA		84.5	-	
Myanmar	57 (swabs)	Chicken	PCR	10.5	8.8	-	Fujisawa <i>et al.</i> , (2019)
Netherlands	173 (serum)	Layer, Broiler, Turkey	SPAT	ND	73	MS seroprevalence was low in grandparent stock layer (0%) and broiler Feberwee <i>et al.</i> , (2008) (10%); In parent stock, layer and broiler was 25% and 35%; Higher in commercial layer stock (73%). Broiler was (6%) and turkey, was 16%	
Nigeria	552 (serum)	Chicken	MG/MS	91.8	ND	-	Ahmed <i>et al.</i> , (2015)
			ELISA				
Nepal	134 (serum)	Chicken	SPAT	23.13	47.76%	-	Yadav & Singh (2018)
			ELISA	17.16			
Pakistan	600 serum	Breeder, broiler, layer	ELISA	65	ND	MG seroprevalence showed highest in layers (75%) followed by breeders (70%) and broilers (50%)	Muhammad <i>et al.</i> , (2021)
	600 swab		Culture	18.8			
	1667 (serum) broiler (n=280), broiler breeder (n=730)	layer (n=657)	SPAT	45.9	40.4	-	Shoaib <i>et al.</i> , (2019)
				7.14			
				59.6			
	358 Serum	Layer	ELISA	29.88	ND	High prevalence in 24-31 wks age group layers (44.17%) as compared to 55-63 wks (14.49%). Seasonal study showed highest prevalence in December (44%) and lowest in October (20%)	Haider <i>et al.</i> , (2019)
			SPAT	20.67			

124 (swabs, tissues)	Broiler, Layer	PCR	40.32	13.70	-	Khattoon <i>et al.</i> , (2018)
380 (tissues)	Layer	Culture	27.6	ND	-	Rauf <i>et al.</i> , (2013)
28694 (Carcasses)	Broiler, Layer	PCR	68.9	-	-	Ahmad <i>et al.</i> , (2012)
		Post Mortem Examination	17.27	-	-	
906 (swabs, tissues)	Layer	Real-time PCR	ND	29.0	-	Kursa <i>et al.</i> , (2019)
		LAMP		25.0		
		PCR		22.0		
450 (serum, swabs)	Chicken	SPAT, ELISA	18.2-40, 16.7-40,	ND	Higher prevalence found in older flock	Pakpinyo <i>et al.</i> , (2007)
		PCR	18.2- 40%			

ND - Not detected

et al., 2016). MS isolates from air sac lesions are abler to cause airsacculitis, and those isolated from synovia are more capable to produce synovitis (Kleven *et al.*, 1975). Airsacculitis caused by of MG and MS is enhanced by either vaccination or infection with respiratory viruses like ND, IB, LPAI and *E coli* (Corstvet and Sadler, 1966; Sato, 1970; Omuro *et al.*, 1971; Kleven *et al.*, 1972; Springer *et al.*, 1974; Gross,1990; Stipkovits *et al.*, 2012; Leigh *et al.*, 2012). Co-infection with IBV can enhance MS arthritis after aerosol exposure (Landman and Feberwee, 2004). The severity of the disease in the case of both MG and MS, is enhanced due to immunosuppression (Vardaman *et al.*, 1973; Giambrone *et al.*, 1977; Bao *et al.*, 2020) and adverse weather and environmental conditions (Sato *et al.*, 1973; Yoder *et al.*, 1977).

CLINICAL SIGNS

The most important clinical signs of natural infection with MG are tracheal rales, nasal discharge, coughing, reduced feed consumption, and loss of weight. Egg production declines but usually is maintained at a lowered level (Ley and Yoder, 2008; Farooq *et al.*, 2020). Keratoconjunctivitis with the swelling of facial skin and the eyelids, increased lacrimation, congestion of conjunctival vessels, and respiratory rales were reported (Nunoya *et al.*, 1995).

The MS infection occurs in two forms, respiratory and synovitis types with or without generalization (Stipkovits and Kempf, 1996). Poor growth and swollen foot are the commonly seen signs in infectious synovitis (Morrow *et al.*, 1990). The initial signs seen in infectious synovitis-affected flocks are pale comb, lameness, and retarded growth. Later on ruffled feathers, shrunk or bluish combs and the appearance of swellings around joints mainly hock joints, foot pad and breast blisters are common. The acute signs are followed by slow recovery, but synovitis may continue for lifetime. Asymptomatic manifestation is very common and air sac lesions from MS infection are most common in winter (Kleven, and Ferguson Noel, 2008). Respiratory signs and enlargement of keel bursa has been reported in broilers (Senties-Cue *et al.*, 2005). Other important pathogenic effects of MS in poultry are amyloid arthropathy in poultry (Landman and Feberwee, 2001) and egg shell apex abnormalities (EAA) (Catania *et al.*, 2010; Ranck *et al.*, 2010; Jeon *et al.*, 2014; Sumitha *et al.*, 2018a). EAA is characterized by a roughened shell surface, shell thinning, increased translucency, cracks and breaks at the top cone of the egg, up to approximately 2 cm from the apex (Feberwee *et al.*, 2009). MS may induce transient immunosuppression, increase in mortality of 1-4%a decrease in egg production and hatch rate (Mohammed *et al.*, 1987; Stipkovits and Kempf 1996).

PATHOLOGY

M. gallisepticum

Commonly observed lesions are catarrhal or frothy exudate in nasal and paranasal passages, trachea, bronchi, and air sacs, cloudiness or thickening of air sacs with caseous exudate accumulation, and deposition of caseous material in the bronchi and lungs (Ley and Yoder, 2008; Islam *et al.*, 2011; Rajkumar *et al.*, 2017a). In complicated CRD associated with high mortality, airsacculitis, fibrinous or fibrinopurulent perihepatitis, and adhesive pericarditis

Table 2. Details of the prevalence/incidence studies of MG and MS carried out in India.

State	Sample type and Number	Bird type	Test	Prevalence/ incidence (%)		Prevalence Pattern		References
				MG	MS	MG	MS	
Andaman and Nicobar Islands	430 (blood)	Village Chicken	SPAT	13.26	ND	-	-	Sundar <i>et al.</i> , (2007)
Chhattisgarh	428 (tissues)	Chicken	PCR	ND	6.30	-	-	Gumasta <i>et al.</i> , (2023)
Haryana	306 (serum)	Chicken	i-ELISA	50.32	61.76	-	-	Yadav <i>et al.</i> , (2021)
			DOT blot assay	41.83	53.92			
	5,664 (Carcasses)	Broiler	CRD	7.94	-	-	-	Mahajan <i>et al.</i> , (1994)
			Incidence by Post mortem					
Kerala	50 (tissues)	Chicken	PCR	2.0	ND	-	-	Sankar <i>et al.</i> , (2012)
Madhya P.	60 (swabs, tissues)	Broiler	CRD	10.61	ND	75% of CRD cases in less than 3 wks age		Poorna Chandhar <i>et al.</i> , (2018)
			detection on PM Examination					
			PCR	13.3				
	98 (serum)	Layer	ELISA	21.4	ND	Seroprevalence was highest in age group 6-24 wks (30%) and was least in 61-77 wks age (12.50%)		Singh <i>et al.</i> , (2016)
Maharashtra	99 (tissues)	Broiler	PCR	7.07	ND	-	-	Vanjari <i>et al.</i> , (2022)
	60 (pooled tissues from 60 flocks)	Broiler	PCR	10.0	20.0	Mixed infection of MG and MS and Concurrent infection with <i>E. coli</i> reported		Bagal <i>et al.</i> , (2019)
Tamil Nadu	790 (tissues)	Breeder, broiler, layer	PCR	13.2	ND	-	-	Manimaran <i>et al.</i> , (2019)
	18 (serum, swabs)	Layer	Culture	11.5				Sumitha & Sukumar, (2018)
			ELISA	ND	100			
			Culture		33.3			
			PCR		77.7			
	184 (serum)	Layer	SPAT	ND	51.1	-	-	Sumitha <i>et al.</i> , (2017)
			ELISA		54.3			
	103 (serum)	Layer	ELISA	53.4	ND	Highest (100%) sero-prevalence recorded in age group 32 wks and the lowest (0%) was recorded in 68 wks		Udhayavel <i>et al.</i> , (2016)
	584 (serum)	Layer	ELISA	62.15	91.60	Higher seropositivity of both MG and MS in adult layer as compared to chicks and growers		Sumitha <i>et al.</i> , (2015)
	43,484 (Carcasses)	Layer	CRD	1.82	-	-	-	Sriithar <i>et al.</i> (1997)
			Incidence by Post mortem					
Andhra P., Karnataka, Tamil Nadu	425 (tissues)	Chicken	PCR	ND	0.7	-	-	Saritha <i>et al.</i> , (2010)
Telangana	13,394 (Carcasses)	Breeder, broiler, layer, indigenous	CRD	11.50	-	Incidence highest in broiler breeder followed by indigenous and layer.		Rajkumar <i>et al.</i> , (2017)
			Incidence by Post mortem			Incidence in chicks, growers and adults were 6.62, 18.52 and 9.25% respectively and 12.89 and 8.04% in males and females		

Different states of country	1285 (serum)	Broiler, breeder	ELISA	32.06	ND	Higher seroprevalence during winter (58.10%) than in summer season (7.43%) and higher in young birds (42.75%) compared to adults (10.46%)	Baksi <i>et al.</i> , (2016)
	1354 (serum)	Broiler, breeder	ELISA	ND	41.1	Higher Sero-prevalence during summer (58.50%) than in winter (15.3%)	Baksi <i>et al.</i> , (2016a)
Haryana, Karnataka, Maharashtra, Telangana, Uttar Pradesh	534 (tissues)	Broiler, layer	PCR	17.94	ND	Concurrent infections with ND, LPAL, IB, <i>E. coli</i>	Kaore <i>et al.</i> , (2018)
Gujarat, Himachal P., Karnataka, Odisha, Tamil Nadu, Telangana, West Bengal	635 (serum)	Layer	ELISA	32.6	52.1	Higher prevalence of both MG and MS in older birds	Rajkumar <i>et al.</i> , (2018)
	309 (swabs)	Breeder	PCR	11.6	33.0		
Andhra P., Bihar, Goa, Gujarat, Haryana, Jharkhand, Karnataka, Kerala, Maharashtra, Odisha, Punjab, Rajasthan, Tamil Nadu, Telangana, Tripura, and West Bengal	3620 (swabs)	Layer, Breeder	PCR	6.43	23.61	-	Giram <i>et al.</i> , (2022)

were reported. Keratoconjunctivitis is reported with edema of the subcutaneous tissue of the face and corneal opacity (Nunoya *et al.*, 1995). Salpingitis has been reported in birds with decreased egg production in MG infected flocks (Domennutb *et al.*, 1967; Nunoya *et al.*, 1997). Gross lesions in the oviduct characterized by the formation of greyish-white caseous plugs partially or throughout the length of the oviduct after yolk sac inoculation of layer chicks with MG were observed (Pruthi and Kharole, 1961).

Microscopic lesions in chickens are characterized by marked thickening of the mucous membranes of respiratory tract due to edema, hyperemia, epithelial and submucosal infiltration with lymphocytes, increased tracheal mucosal thickness, hyperplasia of the mucous glands, cytoplasmic vacuolation in ciliated epithelium and loss of cilia (Kerr and Oslon 1967; Tajima *et al.*, 1979; Charlier *et al.*, 1981; Gaunson *et al.*, 2000; Islam *et al.*, 2011). Cyst formation in the mucosal epithelium and dilatation of mucosal glands with accumulated secretion and foci of lymphoid hyperplasia in the submucosa were also reported. Air sacculitis characterized by perivascular lymphocytic infiltration, lymphofollicular reaction, fibrin exudation, epithelial cell proliferation and lung lesions like congestion, haemorrhages, focal necrosis and lymphocytes and heterophils infiltration in the interlobular septae, lymphofollicular reaction and submucosal proliferation and infiltration of lymphocytes in the secondary bronchi were also reported (Trampel *et al.*, 1981; Islam *et al.*, 2011; Rajkumar *et al.*, 2017a). In chicken with keratoconjunctivitis, epithelial hyperplasia, severe cellular infiltration and edema of the subepithelial and central fibrovascular connective tissue stroma were recorded (Nunoya *et al.*, 1995). In layer chicken with salpingitis, marked thickening of the oviductal mucosa due to epithelial hyperplasia and pronounced lymphoplasmacytic infiltration was observed (Nunoya *et al.*, 1997). Pruthi and Kharole (1961) reported moderate heterophilic infiltration, lymphocytic aggregates, lymphoid follicles, diffuse lymphocytic infiltration and formation of caseous plugs due to fibrin and necrotic cells in the oviduct after yolk sac inoculation of layer chicks with MG.

M. synoviae

MS can give rise to respiratory form of the disease characterised by air sacculitis of different intensities including cloudiness, mild to severe thickening with meaty consistency of air sacs with accumulation of caseous material in the air sacs are seen (Kleven *et al.*, 1972; 1974; Kawakaboo *et al.*, 1980; Lockaby *et al.* 1998). Experimental studies and natural infection with MS have produced swelling of the footpads, hock joints and surrounding tissues with edema and presence of pale yellow viscous exudate along tendons, in joint cavities and hypotarsal and keel bursa, oedema, purulent exudation, formation of granulation tissue and fibrosis followed by caseation of the exudate in the joint cavities. In later stages villi formation in the synovial membrane, thinning and erosion of articular cartilage, leading to complete destruction of the tarsal joint (Chalquest and Fabricant 1960; Kerr and Oslon, 1967; Morrow *et al.*, 1990; 1997; Sentes cue *et al.*, 2005). Pathogenicity depends on the strain of the MS. Systemic infection may involve various organs including liver, spleen and

brain along with nerves, choroids of the eye and skeletal muscle (Kerr and Olson, 1967; 1970; Lockaby *et al.*, 1998).

Varying microscopic lesions were reported in mild to severe grossly affected air sacs. Focal edema with proliferation of capillaries and mononuclear cells in mild cases, fibrin deposition, accumulation of heterophils and necrotic debris on the air sac surface, hyperplasia of epithelial cells, diffuse infiltration of mononuclear cells and caseous necrosis in severe cases were reported (Kleven *et al.*, 1975; Fletcher *et al.*, 1976; Kawakubo *et al.*, 1980). Lymphoid and plasma cell infiltration in the lamina propria of trachea and lymphocytic infiltration in the alveolar septa and around the bronchi, air capillaries, in the bronchial mucosa, infra orbital sinus and nasal mucosa were also reported (Kawakubo *et al.*, 1980; Lockaby *et al.*, 1998).

Acute and chronic arthritis due to MS infection characterised by invasion with heterophils and monocytes and villus formation and presence of lymphofollicular aggregates in the synovium and progressive erosion of articular cartilage and degenerative changes in underlying bone in severe cases were reported (Jordan, 1981). Infiltration of lymphoid cells, plasma cells, and heterophils in the synovial membrane and perisynovial tissue, and hyperplasia of synovial membrane with villous formation were also reported (Kawakubo *et al.*, 1980; Lockaby *et al.*, 1998; Senties Cue *et al.*, 2005). Amyloid arthropathy characterised by amyloid deposits in mainly joints, rarely in liver and spleen were seen in poultry (Landman and Feberwee, 2001). Mild to severe synovitis in keel bursa characterized by infiltration of lymphocytes and plasma cells, lymphoid nodule formations and fibrosis on the surface of the synovium and hypertrophy of the endothelial lining seen in MS infected chicken (Senties Cue *et al.*, 2005).

Ultrastructural changes

Attachment of Mycoplasmas to tracheal mucosal epithelial cells by terminal organelles, distension of mitochondria with loss of cristae, dilated endoplasmic reticulum, cytoplasmic vacuoles of varying sizes, pyknotic nuclei with margination of heterochromatin were the predominant ultrastructural lesions in MG infected birds. (Tajima *et al.*, 1979). Attachment of MG to microvilli and cilia of the oviductal epithelium in chicken with a terminal bleb and condensation of cytoskeletal components in the adjacent cytoplasm Nunoya *et al.* (1997). The ultrastructural study of synovium from MS infected birds showed organisms in the cytoplasm of adipose cells and lipid containing inclusions accumulated in fibroblasts in tendon sheaths and loose connective tissue, proliferation of synovial cells with increased amount of granular endoplasmic reticulum and lipid inclusions (Walker *et al.*, 1978).

DIAGNOSIS OF MG AND MS

Serologic procedures are useful for flock monitoring for control programs and to aid in diagnosis when infection is suspected. Serum Plate Agglutination test (SPAT) is a quick, relatively inexpensive, and sensitive test and efficient in detecting immunoglobulin M (IgM) antibodies and it has been widely used as an initial screening test for flock monitoring and serodiagnosis (Kleven, 1998). SPAT and/or Enzyme-linked immunosorbent assay are used for the initial screening of the flocks, and the hemagglutination-inhibition (HI) test and (ELISA) are also commonly used for confirmation of reactors (Vardaman and Yoder, 1969). SPAT has shown cross-reactivity between MG and MS whereas no cross-reaction was observed with ELISA and HI (Ewing *et al.*, 1996) but better sensitivity was found for SPAT than the ELISA and the HI tests (Avakian *et al.*, 1988). Several types of serodiagnostic tests have been standardized to detect antibodies in serum, respiratory secretions, synovial fluids, oviducts and yolk (Bencina and Bradbury, 1992; Czifra *et al.*, 1995; Kiseok *et al.*, 2010; Yadav *et al.*, 2021). Studies have shown that vaccine strain antigenic epitopes vary from the pathogenic strains and hence appropriate serodiagnostic tests are necessary to screen the post vaccination titre (Noormohammadi, 2002; 2002a).

The isolation and identification of Mycoplasmas are the gold standard for diagnosis, but it is time consuming and laborious. The Mycoplasma pathogens can be isolated from tracheal or air sac exudates, turbinates, lungs, or sinus exudate in mycoplasma broth or agar medium (Kleven, 1998). Advancements in molecular biology have helped in development of several rapid and sensitive detection methods like PCR, which is advantageous due to rapidness and high sensitivity (Pourbakhsh *et al.*, 2010). Probe based methods and PCRs allow direct detection of *Mycoplasma* DNA in tissues, swab samples, and also in culture medias ((Fernandez *et al.*, 1993; Khan and Kleven, 1993; Lauerma *et al.*, 1995). Multiplex PCRs, real-time PCR, random amplified polymorphic DNA (RAPD), arbitrarily primed polymerase chain reactions, are some of the molecular diagnostic procedures developed for the detection of these pathogens. Dual or multiplex PCRs allow simultaneous detection of *Mycoplasma* pathogens (Sprygin *et al.*, 2010; Wang *et al.*, 1997; Lauerma *et al.*, 1995; Fraga *et al.*, 2013). Bacterial isolation, in addition to serological and genetic detection tests, is being used for monitoring MG infection. Molecular genetic assays such as RFLP, high resolution melting (HRM) curve analysis, gene-targeted sequencing (GTS), and multilocus sequence typing (MLST) could help differentiation of vaccine strains and wild types (Lysnyansky *et al.*, 2005; Bayatzadeh *et al.*, 2011; Ghorashi *et al.*, 2013; Armour *et al.*, 2013; Khalifa *et al.*, 2014; Ghanem *et al.*, 2018; Matucci,

et al., 2020).

PREVENTION AND CONTROL

The prevention and control measures mainly target achieving a poultry stock free from infection by following strict biosecurity measures on the farm premises, treatment, and vaccination. As both these pathogens are transmitted vertically and survive asymptotically in the birds till their lifetime, it is highly essential to procure MG and MS-free birds and fertile eggs. It is better to avoid multi aged poultry flocks as they help in retaining these pathogens in the farm premises forever. Treatment of fertile eggs, with heat at 46°C for 12-14 hours or, by antibiotic treatment, either by in ovo injection or by dipping eggs in antimicrobial solutions (Umar *et al.*, 2017). If *mycoplasma*-free status is not achievable, various strategies may be applied to reduce the disease occurrence, production loss, and spread of the infection. Following strict biosecurity measures and regular sanitation are important in preventing the spread. Higher population density enhances the chance for horizontal spread of these pathogens. An effective monitoring system and early diagnosis using serological monitoring, Mycoplasma isolation techniques, and DNA-based detection methods are needed to prevent or control an MG infection or outbreak. Appropriate antimicrobial therapy and vaccination can be considered as long-term interventions to reduce morbidity, mortality, production losses, and pathogen transmission (Kleven and Ferguson-Noel, 2008).

Immunity

Bursa-dependent antibodies are mainly responsible for providing immunity against both these pathogens and serologic response (Vardaman *et al.*, 1973; Adler *et al.*, 1973; Avakian and Kleven, 1990). Chickens inoculated with MG produce IgA, IgM, and IgG and the antibodies in respiratory secretions play an important role in resistance to the pathogen by inhibiting the attachment of the organism to tracheal epithelial cells (Avakian and Ley, 1993). The healthy recovered birds from Mycoplasma clinical disease are found to have immunity, but may also carry the organism (Bencina, and Dorrer; 1984) and can spread pathogens

by horizontal or vertical transmission. Maternal antibody is found in offspring embryos from immunized chickens and it plays a crucial role in reducing in-ovo pathogenicity and in preventing embryo mortality (Levisohn *et al.*, 1985; Bencina *et al.*, 2005). MG initiates a strong cell-mediated immunity by stimulation of natural killer and cytotoxic T cell responses to infection (Chhabra and Goel, 1981; Gaunson *et al.*, 2006). Also MS-H vaccination against MS promotes stronger Th1 response upon challenge with pathogenic strains (Omotaïne *et al.*, 2023). Recent reports show that MG infection induces oxidative stress, causes changes in cytokines activities and structural integrity of Bursa of Fabricius and results in impaired immune function, and also produces a dysregulated immune response in the lungs and airways (Zhang *et al.*, 2020). The infection can cause initially an increased expression of pro-inflammatory cytokines and later on immunosuppression (Miao *et al.*, 2022).

Vaccination

Mycoplasma infection affects the economy of poultry production by causing production loss and ability to induce immunosuppression thereby making birds more susceptible to other respiratory bacterial and viral pathogens. Many vaccines available which targets to protect against clinical disease and lesions, preventing egg production loss, and in reducing vertical transmission (Glisson and Kleven, 1984; Ferguson-Noel *et al.*, 2012). Various method is used to develop attenuated vaccines like serial passage, selection of a naturally attenuated strain, chemical mutagenesis and genetic reconstitution of attenuated strains with virulence associated factors (Gates *et al.*, 2008). Live attenuated MG vaccines are approved and commercially available based on F strain, 6/85, and ts-11 and are very commonly used as aerosol/ eye drop vaccine. F strain vaccine is efficient in preventing air sac infection, respiratory illness, loss of egg production, and egg transmission (Cummings *et al.*, 1986) and it can remain in the upper respiratory tract throughout the life of the chicken (Abd-El-Motelib *et al.*, 1993). F strain can result in clinical outbreak (Ley *et al.*, 1993) and is found to get transmitted to unvaccinated flocks (Khalifa *et al.*, 2014). The ts-11 vaccine strain is an Australian MG isolate attenuated

Table 3. Commercially available vaccines of MG and MS.

Vaccine name	Strain	Manufacturer
Mycovac-L	MG strain MG6/85	MSD Animal Health
Nobilis® MG 6/85		Merck
Vaxsafe® MG	MG strain ts-11	Bioproperties Pty LTD
FVAX-MG	MG strain F	Merck
Poulvac® Myco F		Zoetis United States
CEVAC MG F		Ceva Sante Animale
AviPro® MG-F		Elanco
VAXXON® MG Live	MG K strain	Vaxxinova® International BV
MG-Bac	-	Zoetis United States
Nobilis® MG inac	MG strain S6	Merck
AviPro104 MG	MG strain R	Lohmann Animal Health
BACTERIN		International
VAXXON® MG Inac	MG strain R	Vaxxinova® International BV
Vaxsafe® MS	MS strain H	Bioproperties Property LTD
Nobilis® MS LIVE	MS strain MS1	Merck

by chemical mutagenesis. Both ts-11 and the 6/85, a US strain are not virulent in chickens, and no horizontal transmission between birds has been reported. Major disadvantages of the ts-11 vaccine are the vertical transmission of the *Mycoplasma* and reversion to virulent form (El Gazzar *et al.*, 2011; Armour and Ferguson-Noel; 2015). Another vaccine strain is K strain which offers protection equivalent to commercial F strain and ts-11 vaccines and gives significant protection from air sac and tracheal lesions, as well as colonization with pathogenic R-strain (Ferguson-Noel and Williams, 2015). Many other live vaccines were developed including GT5 and MG 7 derived from the less virulent strain R-high, and the ts-304, a variant of the ts-11 strain (Condello *et al.*, 2020; Gates *et al.*, 2008; Papazisi *et al.*, 2002a) showing better protection against disease. Inactivated oil-adjuvanted suspension containing bacterins are also commercially available which needs to be used by intramuscular or subcutaneous route injections. As the inactivated vaccines do not contain live organisms, there is no risk for egg transmission or reversion to a virulent form. But high cost, individual bird administration and rarely reported localized vaccine reactions are its limitations. A recombinant live virus vectored vaccine rFP-MG has been developed but is less efficacious than MG-Bac and F-strain vaccines (Ferguson-Noel *et al.*, 2012). For vaccination against MS two live attenuated vaccines are available. MS-H Vaccine, a temperature-sensitive live vaccine that was developed by the chemical mutagenesis of an Australian field strain (86079/7NS) can protect from air sac damage and reduce the incidence of eggshell abnormality caused by MS. Another live attenuated vaccine is based on the NAD-independent MS1 which is a spontaneous attenuated strain during *in vitro* passages of the pathogenic field isolate WVU 1853 (Kreizinger *et al.*, 2017). An inactivated MS vaccine has been developed that can induce both cellular and humoral immune responses in broilers and confers a high level of protection upon challenge, demonstrating a potential application in the field (Gong *et al.*, 2020). Details of the commercially available vaccines are given in Table 3.

CONCLUSION AND FUTURE PERSPECTIVE

MG and MS are the most pathogenic avian mycoplasmas affecting chicken and are reported worldwide causing significant economic losses in the poultry industry. The economic loss occurs due to its multiple capacity to affect various systems and the more severe pathology is seen in concurrent infections with other bacterial and viral pathogens. The difficulty in controlling the infection arises due to its ability to get transmitted horizontally and vertically and its ability to persist in infected flocks for their life time. The three main strategies for the control of *Mycoplasma* infections

includes biosecurity and management practices, treatment, and vaccination. The control strategies targeting the prevention of infection are the best to avoid economic loss. Serological tests are useful for the initial screening of flocks, which can be confirmed by direct PCR or culture analysis. Even though multiple live attenuated and inactivated vaccines based on various strains of MG and MS are available, vaccination is not able to provide a consistent level of protection against clinical disease and transmission, and the disease is controlled mainly by antibiotic treatment. The risk of egg transmission, horizontal spread, and reversion to virulence makes the live vaccines disadvantageous. With the growing concern of antimicrobial resistance worldwide, an effective alternative to reduce economic loss is the need of the hour. Multi epitope or recombinant antigen-based vaccines may provide better immunogenicity and protection with no risk of transmission and should be targeted for the effective control of the disease.

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