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Volume 3 | Issue 2 | December 2021 | Page1-46
Veterinary Science Research

Contents

Articles

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Veterinary Science Research
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ARTICLE

The Influence of Newcastle Disease Virus Major Proteins on Virulence

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ABSTRACT

The Newcastle disease virus (NDV) negative-strand RNA genome contains six genes. These genes encode nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) proteins. The six proteins affect the virulence of NDV in different ways, but available information on the six proteins is disparate and scattered across many databases and sources. A comprehensive overview of the proteins determining NDV virulence is lacking. This review summarizes the virulence of NDV as a complex trait determined by these six different proteins.

1. Introduction

Newcastle disease (ND) is an important poultry disease that causes huge economic loss to the poultry industry and is distributed around the world. ND is caused by Newcastle disease virus (NDV), which infects more than 240 species of birds, and is spread mainly through direct contact between infected birds and healthy birds. ND outbreaks were first reported in Java, Indonesia, and Newcastle upon Tyne, UK, in the mid-1920s. Within a couple of years, ND spread rapidly and became a serious poultry epidemic worldwide.

According to the latest classification issued by the International Committee Taxonomy of Viruses (ICTV), NDV is named *Avian orthoavulavirus 1* (AOAV-1), classified in the genus *Orthoavulavirus*, subfamily *Avulavirinae*, family *Paramyxoviridae*. All NDV strains belong to a single serotype but have multiple genotypes. NDV strains are classified into two classes, I and II, based on genome and fusion gene sequence lengths [1]. Class I viruses contain a single genotype with a genome length of 15,198 nucleotides (nt). Class II consists of 15 genetic groups, I–XV, with genome lengths of either 15,186 or 15,192 nt [2]. The viruses of class I are avirulent in general, while class II contains virulent and avirulent strains [3]. Currently, the predominant strains circulating the world belong to genotypes V, VI, and VII of class II, while

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commonly used vaccines are against LaSota and B1 of class II's genotype II, which were both isolated 60 years ago. These commonly used class II vaccines offer partial protection against current circulating NDV. However, birds immunized with the traditional class II vaccines can still be infected with many currently endemic NDV strains. Live attenuated vaccines or inactivated vaccines developed from currently circulating genotype strains provide improved immune protection against current strains, suggesting the importance of developing vaccines against currently circulating genotypes [4].

The NDV genome includes six open reading frames (ORFs), which encode a nucleoprotein (NP), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), a haemagglutinin-neuraminidase (HN), and an RNA-dependent RNA polymerase (L). A W protein and a V protein are generated by RNA editing during P gene transcription. Viral infection is initiated by adhesion of virions to the surface of host cells. Similar to other paramyxoviruses, the viral HN protein binds to sialic acid receptors on target cell surfaces, triggering the F protein to facilitate fusion of the viral envelope with the plasma membrane of the host cell by a pH-independent mechanism [5]. After entry, the virus replicates, transcripts, and translates in the cytoplasm. Finally, all components of the virion are transported to the plasma membrane and bud with the participation of the M protein [6]. Various steps in the NDV infection cycle require the participation of multiple viral proteins, and its virulence is determined by multiple proteins.

2. NP Protein

The NDV NP gene is 489 amino acids (aa) long and is highly conserved within different virulent strains. NP contains two domains: a highly conserved structural N-terminal region (N_{CORE}) and a C-terminal region (N_{TAIL}). The N-terminus 401 aa share high sequence identities among all NDV isolates, while the C-terminal region is highly variable. The glutamic acid residue at position 402 (E402) in the NP's N_{TAIL} is essential for NDV RNA synthesis [7].

NP binds to a viral RNA-dependent RNA polymerase (RdRp) complex, composed of P and L proteins, and facilitates the formation of the active ribonucleoprotein (RNP) complex, which is necessary for transcription and replication [8]. Moreover, NP regulates viral genome transcription and replication by interacting with P alone or with itself [9]. Zhan *et al.* reported that NDV infection upregulated PI3K/Akt/mTOR and p38 MAPK/Mnk1 pathways to promote translation of viral mRNA. They proposed that during NDV infection, the NP protein is essential for the selective cap-dependent translation of viral mRNA by binding to eIF4E [10].

3. P, V, and W Protein

The NDV polycistronic phosphoprotein (P) gene is approximately 1,450 nucleotides long and encodes a P protein of 395 aa [11]. The P protein plays significant roles in genome replication and transcription. The P protein serine and threonine residues are highly phosphorylated and are considered necessary for virus replication and transcription [12]. In addition, the P protein can interact with the L protein to recruit an NP-RNA template to assemble the RNP complex, which is critical for RNA synthesis [9].

Paramyxovirus P genes encode more than one protein via an RNA editing mechanism. The V and W proteins are expressed through this RNA editing process during NDV infection. In general, the presence or absence of non-templated guanosine nucleosides inserted in NDV P gene-derived transcripts at ORF position 484 determines which protein is encoded. An mRNA without the guanosine nucleoside inserted encodes the P ORF, whereas mRNAs with a +1 or +2 frameshift encode the V or W protein, respectively. The P/V/W proteins all have the same N-terminal regions but different C-terminal regions [13]. Previous studies have shown that the editing frequencies for the P, V, and W mRNAs in NDV-infected cells are 68% for P, 29% for V, and 2% for W [14].

In addition to being a P gene derivation product, the V protein is an antagonist of α/β interferon (IFN-α/β), which contributes to NDV virulence. Host cellular innate immune systems become weakened in stable DF-1 cell lines expressing the V protein [15]. Other studies have shown that the V protein can antagonize IFN and suggest that the NDV V protein functional domain inhibiting IFN is in its C-terminal domain and promotes degradation of the signal transducer and activator of transcription 1 protein (STAT1) immediately after STAT1 is phosphorylated [16]. Others have reported that the V protein can upregulate SOCS3 expression via the MEK/ERK pathway to benefit NDV replication [17]. NDV V proteins target MAVS for ubiquitin-mediated degradation, leading to down-regulated expression of the proteins in the downstream IFNs pathway and the promotion of virus replication [18].

Only in recent years has more W protein research began to emerge [19]. We previously investigated W protein length diversity, demonstrated the presence of the W protein in different NDV strains, and identified its subcellular localization [20]. Our latest results show that the W protein has a nuclear export signal (NES) motif. Due

to a nuclear localization signal (NLS), the W protein can be transported into the nucleus via KPNA family proteins, including KPNA1, KPNA2, and KPNA6. The W protein's NES motif facilitates its return to the cytoplasm in a CRM1-independent manner. After deletion of its NES, the W protein remains in the nucleus and cannot be sent back to the cytoplasm. We also found that the location of the W protein affects virulence [21].

4. M protein

The matrix protein is located in the viral envelope's inner surface, and is considered the third NDV envelope protein. Like other paramyxovirus M proteins, the NDV M protein is a nucleocytoplasmic shuttling protein [22]. In addition to viral particle assembly function in the cytoplasm and budding at the cell membrane, the NDV M protein localizes in host nuclei at an early stage after infection and remains there during infection. This M protein nuclear-nucleolar localization is thought to regulate a balance between viral replication and transcription and inhibit host protein synthesis, similar to that seen in the M protein of other negative-sense RNA viruses, including the M protein of the measles virus and in the *Vesicular stomatitis Indiana virus* (VSV) [23]. These studies support the notion that the NDV M protein is an essential multifunctional viral protein that plays an essential role in virus growth and replication.

Among the M proteins of paramyxoviruses, only Sendai virus (SeV) and NDV M proteins have been observed in the nucleoli. A previous study showed that the NDV M protein enters the nucleus and co-locates with the nucleoli through a bipartite NLS independent of other viral proteins [24].

Most paramyxovirus M proteins, including NDV, are largely hydrophobic, but do not have transmembrane properties. This is consistent with observed peripheral attachments of paramyxovirus M proteins to the viral membrane. However, the basic residue distribution in the NDV M protein sequence is not uniform. M protein charge distribution analysis shows that the N-terminal 100 aa are acidic in general, but the remainder are strongly basic. Recent studies have reported that most cellular or viral proteins possess essential residue-rich peptides to mediate nuclear or nucleolar localization [25].

The M protein can interact with a variety of host proteins. In this way the M protein inhibits host protein function and promotes NDV growth and replication. Host protein interactions with M proteins have been demonstrated using co-immunoprecipitation (Co-IP) and a yeast two-hybrid system [26]. The NDV M protein and avian nucleophosmin B23.1 co-localize in the nucleolus in co-transfected HEK-293T cells. The binding of M protein with B23.1 was shown using Co-IP assays in this research as well [27]. Furthermore, an FPIV aa sequence motif occurs as a late domain in the N-terminus of the NDV M protein. Mutation of the phenylalanine or proline within this FPIV motif to alanine results in reduced virus budding and decreased virus replication [28].

5. HN and F Protein

The NDV envelope contains two transmembrane glycoproteins, HN and F. The NDV HN protein is multifunctional, affecting processes of viral adsorption, internalization, replication, and dissemination [29]. The HN protein attaches the virus to sialic acid-containing receptors on the surface of a host cell. HN protein neuraminidase activity (NA) also mediates the cleavage of sialic acid from sugar side chains. The F protein mediates the fusion of the virion envelope with the cellular plasma membrane [30]. Both the HN and F proteins are essential for viral virulence and pathogenicity. As protective antigens, both HN and F proteins can induce the production of virus-neutralizing antibody response [31]. The F protein can protect birds from virulent NDV challenge. The HN protein has also been shown to induce protective immunity against NDV infection in chickens, although the neutralizing antibodies show lower titers. Additionally, monoclonal antibodies against F protein neutralize NDV more effectively than monoclonal antibodies against HN protein [32].

The F protein is a class I transmembrane protein, and the precursor exists in the form of F_0 [33]. The precursor protein F_0 is cleaved by cellular proteases into two subunits, F_1 and F_2. This F_0 cleavage is necessary for virus infection. The transmembrane (TM) domains of viral F proteins play a vital role in NDV F protein fusion activity [34]. The oncolytic activity of NDV has been traced to the minor multifunctional protein HN, and the HN protein has been shown to induce apoptosis in chicken embryo fibroblast cells [35].

The head and stalk region of the NDV HN protein functions in promoting membrane fusion. In this region, tetramer tails have an up process, which is called the hinge structure [36]. The role of the highly conserved amino acids in the head region of the HN protein is critical, and changing various amino acids in this region disables the F-promoting ability of the HN protein and inhibits syncytia formation. An analysis of HN globular head structure shows that the NA activity site is associated with a β-sheet propeller motif [37]. The binding of the HN stalk region to sialic acid promotes an F protein conformational change and activates the fusion of the viral envelope with

cell membranes [38].

Avirulent NDV strains, including Queensland V4, Ulster, and D26, possess HN proteins 616 aa long with an additional 39 or 45 aa at the C-terminus. These HN proteins appear to be precursors that can become bioactive after the removal of additional residues at the C-terminus [39]. In contrast, most NDV virulent strains have HN protein lengths of 577 or 571 aa. Hence, no residue removal is required for these HN proteins for full biological function. Notably, the shortest HN protein length observed is 571 aa, and this has only been found in velogenic and mesogenic strains. Therefore, the length of the NDV HN protein is assumed to closely relate to the virulence of the virus [40].

An NDV lentogenic strain, LaSota-based recombinant virus was generated with the 45 aa extension in the HN protein C-terminus, and this C-terminus extension was shown to not determine NDV enteric tropism [41]. However, recombinant virus virulence was decreased in this study compared with that of its parental virus, suggesting that the additional HN protein C-terminus 45 aa may be involved in virulence regulation. In another study of C-terminal extension effects, a recombinant virus with a C-terminus 45 aa insert derived from the lentogenic PHY-LMV42 strain was introduced into the mesogenic NDV Anhinga strain and rescued by reverse genetics techniques. The virulence of the recombinant virus and its parental strain was determined by mean death time (MDT) and an intracerebral pathogenicity index (ICPI). Their results indicated that the HN protein C-terminal extension may reduce virulence in virulence deficient NDV strains [42]. We also studied effects associated with NDV HN protein length diversity and biological properties. Our results show that NDV tissue tropism, replication ability, and pathogenicity are all closely associated with the origin of the HN protein [43].

6. L Protein

The L protein is 2,204 aa long with a molecular weight of 250 kDa. It is the largest NDV protein, containing almost all catalytic activities related to viral replication, transcription, and translation. The L gene may enhance virulence by improving viral gene replication levels. Recombinant L gene replacement chimeric viruses were compared with the parental virus, and significant changes in biological characteristics reveal that the L protein has a particular effect on virulence [44]. We previously demonstrated that NDV's envelope-associated proteins contribute significantly to genotype VII NDV virulence, and its polymerase-associated proteins also have a significant effect on viral pathogenicity. In the same research we showed that the L protein is a significant contributor to NDV virulence among the polymerase-associated proteins when combined with homologous NP and P proteins [45]. Notably, NP encapsulates the entire genomic RNA and the L protein works as an RdRp, combining with the P protein to form a complex that recognizes RNP for transcription and replication [46]. However, those specific domains of the L protein that determine virulence remain to be investigated.

The L protein, as a multifunctional protein in the process of virus proliferation, plays several vital roles. Due to its conservation, the L protein has become the first target protein to be investigated for antiviral drugs in other paramyxoviruses.

7. Conclusions

More and more studies have illustrated that NDV virulence is determined by the single and/or multiple roles of both structural and non-structural proteins. For NDV, the multi-basic amino acid cleavage motif of the F protein is an indispensable prerequisite for virulence. The other five structural proteins are also critical to virulence. Furthermore, other factors such as 5′ untranslated regions (UTRs) or intergenic sequences (IGSs) are involved in determining the virulence of NDV [47]. However, we must note that different and various contributions from these virulence factors may be associated with particular strains. As seen with velogenic strains, the F protein cleavage site is much more important than all other factors including the replication complex and noncoding regions. Another point to consider is the validity of using the ICPI as a criterion for determining virulence [46]. In conclusion, this review discusses NDV viral protein functions and those proteins' effects on virulence. An enormous amount of knowledge about ND and its causative virus has been obtained and deduced recently, as befits such a significant disease. However, it remains as an influence on the worldwide poultry industry, either as a continuing disease problem or as a constant threat.

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ARTICLE

Survey on Avian Malaria Parasites in Village Chickens (*Gallus gallus domesticus*) in Gombe Local Government Area, Gombe State, Nigeria

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ABSTRACT

Reports of avian malaria parasites in village chicken in Nigeria generally remain fragmentary and scarce. The study was conducted in Gombe Local Government Area of Gombe State, Nigeria to investigate avian malaria parasites in Village Chickens (*Gallus gallus domesticus*) and to determine the risk factors associated with the prevalence of the haemoparasites. A total of 530 village chickens blood samples were obtained from apparently healthy village chickens' brachial veins using sterile 2mls syringes and 23 gauge needles. Thin blood smear was made from each blood sample, and Giemsa stained and examined for the presence of avian haemoparasites under an electro-microscope. The result indicates 23.8% overall prevalence rate of three species of avian malaria parasites consisting of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species. *Plasmodium* spp. has the highest prevalent rate of 13.0% followed by *Haemoproteus* spp. (5.1%), mixed *Plasmodium s*pp. + *Haemoproteus* spp. (4.9%) infection and *Leucocytozoon* spp. (0.8%). Prevalence of avian malaria parasites was significantly higher in cocks compared to hens ($p < 0.05$), as well as higher in adults compared to growers chickens ($p < 0.05$). This study also showed a higher prevalence of avian malaria parasites during the rainy season compared to the dry season of the study period. It was concluded that haemoparasites of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species that occur in both single and mixed infections are prevalent among village chickens that are apparently healthy in Gombe Local Government Area of Gombe State, Nigeria.

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1. Introduction

Among the village poultry species, village chickens also known as 'domestic fowl, rural chickens or local chickens' are the most predominant in Africa's rural poultry sector [1]. Village chickens are typically found in nearly every rural household and raised extensively, with little or no particular attention in terms of husbandry and health care [2,3]. They are commonly found to scavenge around households in unhygienic areas, thus being predisposed to disease vectors like parasitic infection [4,5]. Diseases are regarded as one of the most important constraints for the productivity of village chickens in most developing African countries including Nigeria [3]. The effects of parasitism are often severe on birds [6]. Blood parasites have been reported to influence the evolution and ecology of many bird species [5,7], and are distributed across a wide variety of habitats and geographical regions [8].

Blood parasites have a complex life cycle, usually involving a vector that transmits the infection [9-11]. The frequency and distribution of these vectors varies between habitats, primarily depending on the weather [12]. Several researches dealt with the geographic distribution of genetically distinct blood parasites in different regions and ecosystems [13-15].

Taxonomists have described more than 200 species of avian haemosporidians of hundreds of bird species studied worldwide and categorized them into four distinct genera, *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Hepatocystis* [16,17]. In chickens, the most endemic haemoparasites are *Plasmodium, Haemoproteus* and *Leucocytozoon* [18].

In birds, haemosporidian species can be detected and identified using the method of microscopy that has been considered the "gold standard" diagnostic tool for haemoparasites [9,19], and by amplifying and sequencing DNA [8].

In general, few researches have been documented into the prevalence of avian haemoparasites in poultry species in certain parts of Nigeria [20,21] and other developing countries [22,23]. The most recent research focused on the identification of avian haemoparasites in wild birds [18,24,25]. Studies of the genera of haemoparasites infecting village chicken in Nigeria, particularly in the northeastern region, remain fragmentary and scarce, hence the need to conduct the present study. Therefore, the objective of the study is to determine the genera of haemoparasites that causes avian malaria in village chickens in Gombe Local Government Area of Gombe State, Nigeria.

2. Materials and Methods

Study Area

This study was carried out in Gombe Local Government Area, which is the capital and largest city of Gombe State located in Northeastern Nigeria (Figure 1). Gombe state is located between latitude 9^0 $30^{'}$ and 12^0 $3^{'}$ N and longitude 8^0 45' and 11^0 $45^{'}$ E [26]. The total poultry population in Gombe State is approximately 508, 305 comprising 462,000 backyard poultry and 46,305 exotic poultry [27]. Village poultry farmers' households and live birds markets within the study area were visited for blood sample and other data collection.

Sampling Period

Blood sampling was carried out from apparently healthy during the study period from the month of February, 2019 and January, 2020, within two (2) seasons *viz:* the rainy season (April - September) and dry season (October - February). All study locations were visited for blood samples collection on alternate periods within these study periods.

Study and Target Population

The study population of the present study was village chickens of both sexes and two age groups. Sex of birds, was determined based on their morphology breeding status and plumage. Ages of birds were determined using the maturity of the beak and information from poultry farmers. The age was categorized into adult (above 5 months old) and growers (between 3 - 4 months old). Source of birds during the period of blood sampling were noted (farmer household or live birds markets).

Ethical approval

Ethical approval for the present study was duly obtained from and approved by the Institutional Animal ethics and Research committee of the Faculty of Veterinary Medicine, University of Maiduguri, Maiduguri, Borno State, Nigeria. After obtaining the consent of poultry farmers and sellers to sample their chickens, the village chickens were caught and caged to rest overnight and prepared for specimen and data collection very early in the morning. Selected village chickens were gently grabbed by the shanks and manually restrained with caution not to allow the chicken go through neither unnecessary struggle nor stress.

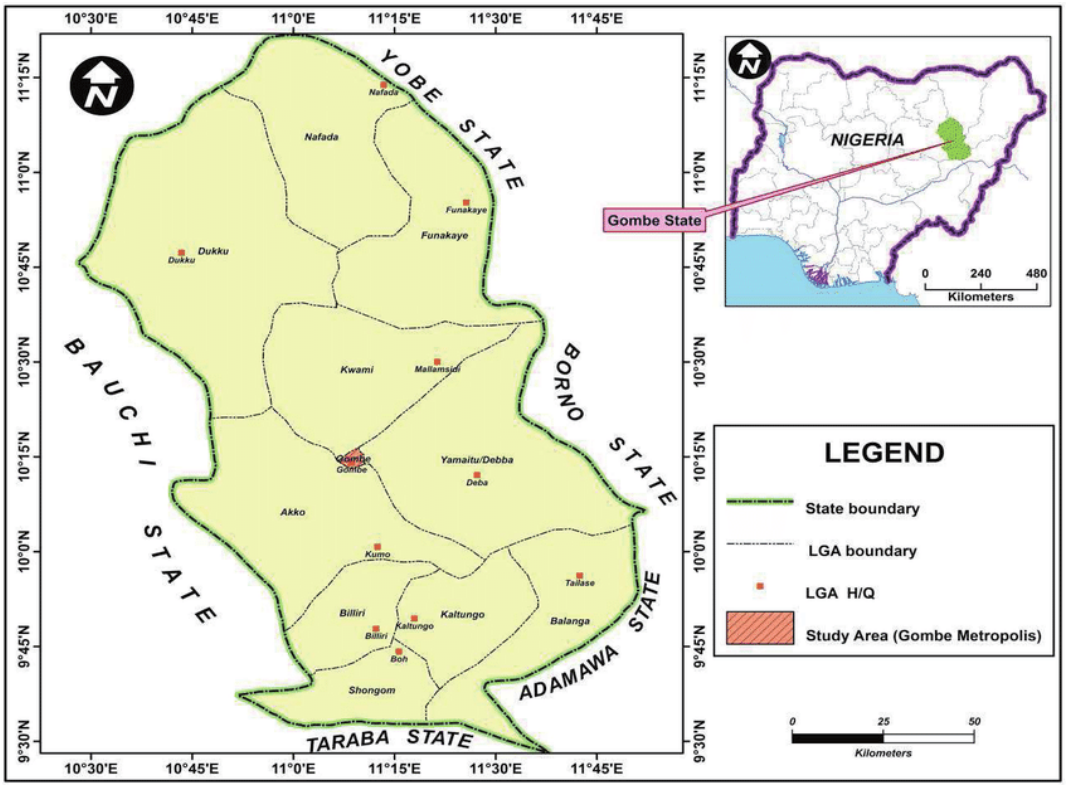

Figure 1. Map of Gombe State showing the study area

Sampling method for birds

Non-probability convenience sampling method was employed. Approximately 3-4 ml of blood samples were obtained aseptically directly from the brachial vein (wing vein) using a 5 ml sterile syringe and 23 gauge needle from each sampled chicken. Each blood sample was immediately dispensed into a sample bottle containing anticoagulant EDTA. All samples were correctly labeled and transported in ice pack container to the Department of Veterinary Parasitology and Entomology Research Laboratory, University of Maiduguri

Identification of Haemoparasites, Parasitological Examination and Detection of Haemoparasites

Thin blood and buffy coats smears of each blood sample were made on two separate slides and left to air dry for a few minutes, then labeled appropriately. The slides were fixed with methanol for five (5) minutes and allowed to air dry, packaged and then stained with diluted 10% Giemsa stain as defined by Zajac and Conboy [28]. The slides were later examined using a microscope at low magnification (x40) and at high magnification (x100) under oil immersion for 10-15 min for the presence of intracellular blood parasites gametocytes as previously described by Valkiūnas [9]. Parasite identification was based on the morphology, height and pigmentation of the endoerythrocytic parasites and photographs compared to the species already described by Taylor *et al.* [29].

Data Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Inc., San Diego, CA). The prevalence rate was calculated as the ratio of the number of hosts infected by one or more parasite species to the total number of hosts examined as described by Bush *et al.* [30]. Chi-square test / Fisher exact tests were used to compare the proportions obtained for the presence or absence of avian haemoparasites infection and the association between independent variables such as study location, age, sex and season. Differences were considered significant at $p < 0.05$ [31].

3. Results

Out of 530 village chickens blood samples examined, 126 chickens were found positive for one or more avian haemoparasites. Hence, the overall prevalence of avian malaria parasites was 23.8% (95% confidence interval was 20.3% - 27.6%). The results also showed that the prevalence rate of avian haemoparasites was higher in village chickens sampled from poultry farmers'

households (14.9%) compared to those sampled from live birds markets (8.9%) within the study area. Although, the difference in the prevalence rates was not statistically significant (p-value = 0.1394; $\chi^2 = 2.185$) as shown in Table 1.

Table 2 presents the results of prevalence of various species of avian malaria parasites in village chickens in the study area. Three (3) different species of avian malaria parasites, namely *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp., were found in single or mixed infections and with different prevalence in infected chickens. The prevalent rate was highest for *Plasmodium* spp. (13.0%; 95% Confidence Interval = 10.4% - 16.2%) followed by *Haemoproteus* spp. (5.1%; 95% Confidence Interval = 3.5% - 7.3%), mixed *Plasmodium* spp. + *Haemoproteus* spp. infection (4.9%; 95% Confidence Interval = 3.4% - 7.1%) and lowest for *Leucocytozoon* spp. (0.8%; 95% Confidence Interval = 0.3% - 1.9%).

The prevalence of different species of avian malaria parasite based on the study location was shown in Table 3. The prevalence of *Plasmodium* spp. in sampled village chickens from household of poultry farmers (7.7%) was higher than in sampled chickens from live bird markets (5.3%), the difference in their prevalence rates was not statistically significant (p-value = 0.7070). *Haemoproteus* spp. prevalence in sampled village chickens from the household of poultry farmers (2.1%) was lower than in sampled chickens from live bird markets (3.0%), difference in the prevalence rate was not statistically significant (p-value = 0.1316). Moreover, the prevalence of *Leucocytozoon* spp. in village chickens sampled from the household of poultry farmers (0.6%) was found to be higher than in chickens sampled from live bird markets (0.2%), the difference in the prevalence rate was also not statistically significant (p-value = 0.6366). The prevalence of mixed *Plasmodium* spp. and *Haemoproteus* spp. infection in village chickens sampled from the household of poultry farmers (3.0%) was higher than in chickens sampled from live bird markets (1.9%), and difference in the prevalence rate was not statistically significant (p-value = 0.7507).

The association of avian malaria parasites species with sex of village chickens in the study area were summarized in Table 4. The findings of this study revealed significantly (p-value = 0.0003) higher prevalence of *Plasmodium* spp. in male (9.6%) compared to the females (3.4%) chickens. The prevalence of *Haemoproteus* spp. was also higher in male (4.0%) compared to female (1.1%) chickens, and the association was statistically significant (p-value = 0.0092). Moreover, the association between sexes of chickens and the prevalence of *Leucocytozoon* spp. revealed no significant statistical (p-value = 0.1260) difference even though the parasite was only detected in the male (0.8%) and none in the female (0.0%) chickens. However, the association between the prevalence of mixed *Plasmodium* spp. and *Haemoproteus* spp. infection and sexes of chickens revealed significantly (p-value = 0.0009) higher prevalence in the male (4.2%) compared to female (0.8%) chickens.

Table 5 summarized the results of age - specific prevalence of avian malaria parasites infections of village chickens in the study area. The results revealed significantly (p-value = 0.0050) higher prevalence of *Plasmodium* spp.in adult (10.2%) compared to grower (2.8%) chickens. The prevalence of *Haemoproteus* spp. was also found to be higher in adult (4.3%) compared to grower (0.8%) chickens, and the association was statistically significant (p-value = 0.0132). However, the association between the prevalence of *Leucocytozoon* spp. and age group of chickens revealed no significant statistical (p-value = 0.3025) difference even though the parasite was only detected in the adult (0.8%) chickens but none in the grower (0.0%). Moreover, the association between the prevalence of mixed *Plasmodium* spp. and *Haemoproteus* spp. infection and age group of chickens revealed significantly (p-value = 0.0035) higher prevalence in the adult (4.3%) compared to grower (0.6%) chickens.

Table 6 summarizes the results of the association between the prevalence of avian malaria parasites infection in village chickens and the season of sample collection in the study area. The results revealed higher prevalence of *Plasmodium* spp. in the rainy (10.0%) compared to dry season (3.0%), and the association was statistically (p-value < 0.0001) significant. The findings of this study also showed significantly higher (p-value = 0.0288) prevalence of *Haemoproteus* spp. in the rainy (3.6%) compared to dry (1.5%) season. In addition, the results of this study also revealed 0.8% prevalent rate of *Leucocytozoon* spp. during the rainy season, whereas *Leucocytozoon* spp. was not detected in the blood samples collected and examined during the dry (0.0%) season, the association between the prevalence and season was considered not statistically significant (p-value = 0.0572). The association between the prevalence of mixed *Plasmodium* spp. and *Haemoproteus* spp. infection and season revealed significantly (p-value = 0.0010) higher prevalence in the rainy (4.0%) compared to dry (0.9%) season.

Table 1. Overall Prevalence of Avian Malaria Parasites in Village Chickens (*Gallus gallus domesticus*) in Gombe Local Government Area, Gombe State, Nigeria

Sampling Location	Number Examined	Number (%) Infected (95% CI)	Prevalence (%)	*p*-value	χ^2	Relative Risk
Live Birds' Markets	230	47 (20.4) (15.7 - 26.1)	8.9[a]	0.1394	2.185	1.080
Poultry Farmers' Households	300	79 (26.3) (21.7 - 31.6)	14.9[a]			
	530	126 (23.8) (20.3 - 27.6)	23.8			

Table 2. Prevalence of Avian Malaria Parasites Encountered in Village Chickens (*Gallus gallus domesticus*) in Gombe Local Government Area, Gombe State, Nigeria

Avian Malaria Parasites Encountered	Number Infected (N = 530)	Prevalence (%)	95% Confidence Interval Lower Limit - Upper Limit
Plasmodium spp.	69	13.0	10.4 - 16.2
Haemoproteus spp.	27	5.1	3.5 - 7.3
Leucocytozoon spp.	4	0.8	0.3 - 1.9
Plasmodium spp. + *Haemoproteus* spp.	26	4.9	3.4 - 7.1
Total	126	23.8	20.3 - 27.6

N = Number of samples examined

Table 3. Study location - Specific Prevalence of Avian Malaria Parasites in Village Chickens (*Gallus gallus domesticus*) in Gombe Local Government Area, Gombe State, Nigeria

Avian Malaria Parasites	Study Location	Number Examined	Number (%) Infected (95% CI)	Prevalence (%)	*p* - value	χ^2	Relative Risk
Plasmodium spp.	Live birds Markets	230	28 (12.2) (8.6 - 17.0)	5.3[a]	0.7070	0.1413	1.017
	Poultry Farmers households	300	41 (13.8) (10.2 - 18.0)	7.7[a]			
Haemoproteus spp.	Live birds Markets	230	16 (7.0) (4.3 - 11.0)	3.0[a]	0.1316	2.274	0.9658
	Poultry Farmers households	300	11 (3.7) (2.1 - 6.5)	2.1[a]			
Leucocytozoon spp.	Live birds Markets	230	1 (0.4) (0.1 - 2.4)	0.2[a]	0.6366	-	1.006
	Poultry Farmers households	300	3 (1.0) (0.3 - 2.9)	0.6[a]			
Plasmodium spp. + *Haemoproteus* spp.	Live birds Markets	230	10 (4.4) (2.4 - 7.8)	1.9[a]	0.7507	0.1010	1.010
	Poultry Farmers households	300	16 (5.3) (3.3 - 8.5)	3.0[a]			

[a,b] Different superscripts indicate significant ($p < 0.05$) difference in study location-specific prevalence; χ^2 = Chi-square

CI = Confidence Interval

Table 4. Sex - Specific Prevalence of Avian Malaria Parasites in Village Chickens (*Gallus gallus domesticus*) in Gombe Local Government Area, Gombe State, Nigeria

Avian Malaria Parasites	Sex	Number Examined	Number (%) Infected (95% CI)	Prevalence (%)	*p* - value	χ^2	Relative Risk
Plasmodium spp.	Male (Cock)	280	51 (18.2) (14.1 - 23.2)	9.6[a]	0.0003	13.193	0.8813
	Female (Hen)	250	18 (7.2) (4.6 - 11.1)	3.4[b]			
Haemoproteus spp.	Male (Cock)	280	21 (7.5) (5.0 - 11.2)	4.0[a]	0.0092	-	0.9477
	Female (Hen)	250	6 (2.4) (1.1 - 5.1)	1.1[b]			
Leucocytozoon spp.	Male (Cock)	280	4 (1.4) (0.6 - 3.6)	0.8[a]	0.1260	-	0.9857
	Female (Hen)	250	0 (0.0) (0.0 - 1.5)	0.0[a]			
Plasmodium spp. + *Haemoproteus* spp.	Male (Cock)	280	22 (7.9) (5.3 - 11.6)	4.2[a]	0.0009	-	0.9364
	Female (Hen)	250	4 (1.6) (0.6 - 4.0)	0.8[b]			

[a,b] Different superscripts indicate significant ($p < 0.05$) difference in sex-specific prevalence; χ^2 = Chi-square; CI = Confidence Interval

Table 5. Age - Specific Prevalence of Avian Malaria Parasites in Village Chickens (*Gallus gallus domesticus*) in Gombe Local Government Area, Gombe State, Nigeria

Avian Malaria Parasites	Age Group (months)	Number Examined	Number (%) Infected (95% CI)	Prevalence (%)	*p* - value	χ^2	Relative Risk
Plasmodium spp.	Adult (> 5)	330	54 (16.4) (12.8 - 20.7)	10.2[a]	0.0050	7.875	0.9042
	Grower (3 - 4)	200	15 (7.5) (4.6 - 12.0)	2.8[b]			
Haemoproteus spp.	Adult (> 5)	330	23 (7.0) (4.7 - 10.2)	4.3[a]	0.0132	-	0.9493
	Grower (3 - 4)	200	4 (2.0) (0.8 - 5.0)	0.8[b]			
Leucocytozoon spp.	Adult (> 5)	330	4 (1.2) (0.5 - 3.1)	0.8[a]	0.3025	-	0.9879
	Grower (3 - 4)	200	0 (0.0) (0.0 - 1.9)	0.0[a]			
Plasmodium spp. + *Haemoproteus* spp.	Adult (> 5)	330	23 (7.0) (4.7 - 10.2)	4.3[a]	0.0035	-	0.9445
	Grower (3 - 4)	200	3 (1.5) (0.0 - 1.9)	0.6[b]			
Total		530	26 (4.9) (3.4 - 7.1)				

[a,b] Different superscripts indicate significant ($p < 0.05$) difference in age-specific prevalence; χ^2 = Chi-square; CI = Confidence Interval

Table 6. Season - Specific Prevalence of Avian Malaria Parasites in Village Chickens (*Gallus gallus domesticus*) in Gombe Local Government Area, Gombe State, Nigeria

Avian Malaria Parasites	Season	Number chickens examined	Number chickens infected	Prevalence (%)	χ^2	P - value	Relative Risk
Plasmodium spp.	Dry	270	16 (5.9) (3.7 - 9.4)	3.0[a]	23.192	< 0.0001	1.182
	Rainy	260	53 (20.4) (15.9 - 25.7)	10.0[b]			
Haemoproteus spp.	Dry	270	8 (3.0) (1.5 - 5.7)	1.5[a]	-	0.0288	1.047
	Rainy	260	19 (7.3) (4.7 - 11.1)	3.6[b]			
Leucocytozoon spp.	Dry	270	0 (0.0) (0.0 - 1.4)	0.0[a]	-	0.0572	0.9846
	Rainy	260	4 (1.5) (0.6 - 3.9)	0.8[a]			
Plasmodium + *Haemoproteus* spp.	Dry	270	5 (1.9) (0.8 - 4.3)	0.9[a]	-	0.0010	1.068
	Rainy	260	21 (8.1) (5.4 - 12.0)	4.0[b]			

[a,b] Different superscripts indicate significant ($p < 0.05$) difference in season-specific prevalence; χ^2 = Chi-square; CI = Confidence Interval

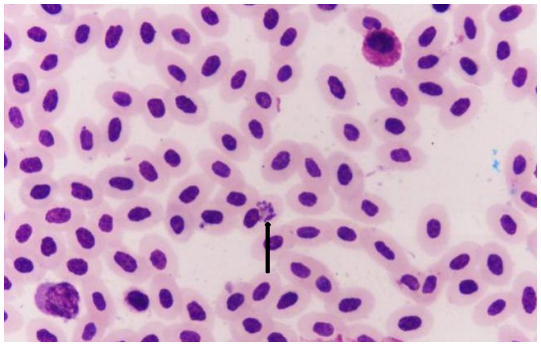

Figure 2. Chicken blood smear showing RBC infected with *Haemoproteus* species. Pigmented gametocytes curving around the nucleus of a mature erythrocyte (arrow)

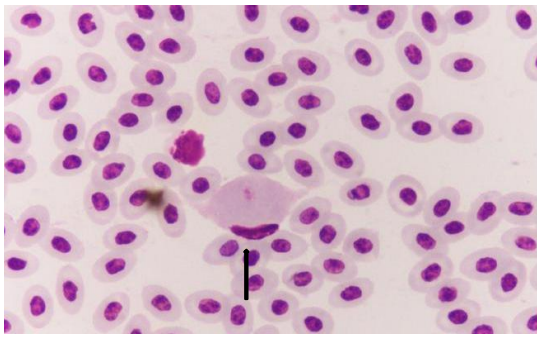

Figure 3. A photomicrograph showing *Leucocytozoon* infection in blood smear (100x). Large basophilic organisms seen distending the avian WBCs (note malformed nucleus of host cell) (arrow)

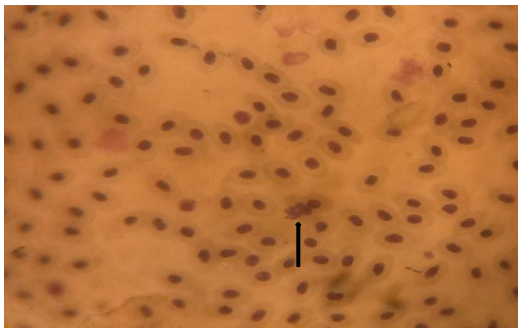

Figure 4. Chicken blood smear showing RBC infected with *Plasmodium* species. Pigmented gametocytes are present within the cytoplasm of mature erythrocytes (arrow)

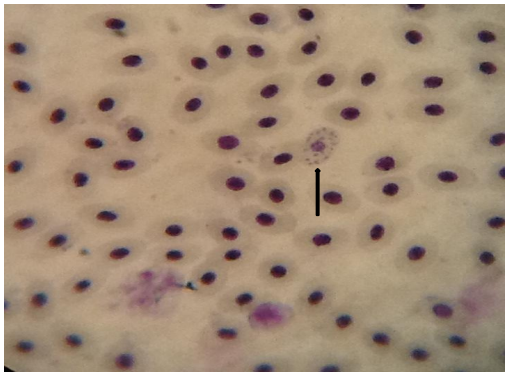

Figure 5. Chicken blood smear showing RBC infected with *Plasmodium* species. Pigmented gametocytes are present within the cytoplasm of mature erythrocytes (arrow)

4. Discussion

The detection of haemosporidians that causes avian malaria in 23.8% of village chickens examined in this study indicated the presence of the parasites and abundance of arthropod vectors capable of transmitting them to scavenging village chicken flocks in the study area. From the result of the present study, the prevalence of avian malaria parasites was higher in village chickens sampled at farmers' households (14.9%) compared to those sampled from the live birds markets (8.9%). Although, the association between the prevalence and sample location was not statistically (p-value = 0.1394) significant. This finding indicated equal chances of getting infection from the two sample locations. However, the major reasons that might be responsible for higher prevalent rates from households may be associated with scavenging nature of the chickens in unhygienic environment, inadequate husbandry and management systems which usually predispose the chickens to high chance of bites from several blood sucking arthropods capable of transmitting haemoparasites including avian malaria, compared to movement restriction of chickens in live birds markets. These findings buttress previous reports of Igbokwe *et al*. [20] and Ogbaje *et al*. [32] who have also reported high prevalent rate of avian haemoparasites in free range chickens compared to chickens sampled from live birds' markets or chickens in hygienic environment. Moreover, the detection of avian malaria parasites in apparently healthy village chickens from live birds markets may not be unexpected, because the chickens are in most instances sourced directly from poultry farmers households and when they gets to the live birds markets, there are usually no discriminations of health status or screening for diseases before mixing chickens from different sources.

The present study discovered three species of avian malaria parasites, namely *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp., infecting village chickens in the study area. This finding indicates the presence of sufficient blood sucking arthropod vectors capable of transmitting these parasites to village chickens and probably to other domesticated poultry species in the study area. The findings from this research agree with those of Walther *et al*. [33] and Nourani *et al*. [34] who identified these three avian haemosporid parasites as the most prevalent haemoparasites in birds. The present study has also found mixed *Plasmodium* spp. and *Haemoproteus* spp. infections in infected chickens. Discoveries from this study supported previous findings from Kenya [23], Malaysia [35], Iraq [36], Malawi [37], Bangladesh [38], Pakistan [39] and Benue, Nigeria [32]; where these three species of avian malaria parasites were also reported in various similar studies in free range chickens. In some parts of Nigeria, only *Plasmodium* spp. were identified from Maiduguri and Sokoto by Igbokwe *et al*. [20] and Usmana *et al*. [40], respectively. In another study, Opara *et al*. [41] reported only *Leucocytozoon* while Opara *et al*. [42] reported *Plasmodium* and *Microfilaria* in Imo State in scavenging chickens. Still from Nigeria, Karamba *et al*. [43] and Lawal *et al*. [44] published on *Plasmodium* spp. and *Haemoproteus* spp. from Kano and Maiduguri respectively. While from other parts of Africa, *Plasmodium*, *Leucocytozoon*, *Aegyptianella* sand *Trypanosoma* species have been identified in village chickens from Zimbabwe [45] and Ethiopia [46]. In addition, Poulsen *et al*. [47] and Njunga [48] have reported *Plasmodium* and *Aegyptianella* species in scavenging chickens from Ghana and Malawi respectively. Gimba *et al*. [35] found *Plasmodium, Haemoproteus, Leucocytozoon, Microfilaria* and *Trypanosoma* species in village chickens in Malaysia, while Hasan *et al*. [49] reported *Plasmodium* and *Leucocytozoon* species in village chickens in Bangladesh. The variation in the species of haemoparasites discovered in village chickens from different studies could be influenced by the topographical factors, availability and abundance of arthropod vectors responsible for the transmission of the parasites, as well as difference in husbandry and management systems of chickens. In addition, other suggestive reasons that may influence the pervasiveness of avian haemoparasites infection may include season of sampling, sampling effort, differences in habitat and climate [19,50].

Amongst the three species of haemoparasites detected in the present study, *Plasmodium* species (13.0%) was the most prevalence followed by *Haemoproteus* (5.1%), mixed *Plasmodium* spp. and *Haemoproteus* spp. (4.9%) while the prevalence of *Leucocytozoon* spp. (0.8%) was the least in the infected village chickens. The finding of *Plasmodium* species as the most prevalent haemoparasites in this study may be correlated with the abundance of mosquitoes in the study area being the common vectors of *Plasmodium* species. This finding concurs with previous reports which have discovered that several families of blood sucking arthropods vectors including mosquitoes which are capable of transmitting avian malaria are highly prevalent in the Northeastern Nigeria including the present study area [20,51]. Zhang *et al*. [18], Sabuni *et al*. [23], Nath and Bhuiyan [38], Etisa *et al*. [46], Hasan *et al*. [49], Sadiq *et al*. [52] and Shadan [53], who described *Plasmodium* species in their different study areas as the most encountered avian haemoparasites in scavenging village chickens, supported

by the findings of the current study. The findings of the present study contrast those of Hasson [36] and Permin *et al*. [45] who reported *Haemoproteus* species, Nath and Bhuiyan [38] who reported *Leucocytozoon* species and Hasson [36] who reported mixed *Plasmodium* and *Heamoproteus* species infection as the most common avian haemoparasites in village chickens in their various studies. Variation in the sampling season, abundance of vectors, method of diagnosis, efforts of sampling, geographical factors, variation in ecological and climatic factors as well as variation in the management and husbandry systems used in the rearing of village chickens may be attributed to the explanation for disparity in the recorded prevalent rates and incidence of avian haemoparasites in village chickens.

In the current study, the prevalence of *Plasmodium* (13.0%) species found in village chickens is greater than 11.4% recorded in Maiduguri, Borno State Northeastern Nigeria [20] and 12.0% in Sokoto, Sokoto State Northwestern Nigeria [40], but lower than 33.3% in Owerri, Imo State Southeastern Nigeria [42] and 32.0% in Ibadan, Oyo State Southwestern Nigeria [52]. Some parts of Africa have recorded varying prevalence rates of *Plasmodium* species that are higher than the results of this current study. In Ghana, Poulsen *et al.* [47] reported a prevalence rate of 27%, 18.2% have been reported in Ethiopia [46], 15% in Malawi [48], 14.9% in Zimbabwe [45], Mbuthia *et al*. [54] and Sabuni [22] reported 29.8% in Kenya.

The finding of the present study also showed a prevalence rate of 5.1% of *Haemoproteus* species in village chickens. This finding is higher than 1.3%, 0.8%, 0.9% and 2.5% reported by Sadiq *et al.* [52], Gimba *et al.* [35], Sabuni *et al.* [23] and Nath and Bhuiyan [38] from Ibadan, Malaysia, Ethiopia and Bangladesh respectively. In the present study, the prevalence of *Haemoproteus* species recorded is lower than 23.3% reported in Bangladesh [55] and 13.2% in Iraq [36]. The disparity in geographic distribution, management systems, and vector abundance may be some of the reason for these differences in prevalence rates.

The result of the present study revealed 4.9% prevalent rate of mixed *Plasmodium* spp. and *Haemoproteus* spp. infection. This finding is higher than 3.5% and lower than 47.4% reported by Sabuni *et al.* [23] and Hasson [36] respectively.

The result of the present study showed a prevalence of 0.8% of *Leucocytozoon* spp. in village chickens in the study area. This finding is lower than 20.0% reported in Ibadan [52] and 8.9% in Owerri [41]. Compared to other haemoparasites, the low prevalence of *Leucocytozoon* spp. in village chickens found in the present study may be due to the sparse abundance of arthropod vectors capable of transmitting the parasites in the study area. In some parts of Africa, higher prevalence of the *Leucocytozoon* species has previously been reported from village chickens, such as 31.6% in Kenya [54], Sabuni *et al*. [23] reported 31.6% in Ethiopia, Permin *et al*. [45] reported 4.3% from Zimbabwe, while Sabuni [22] and Sehgal *et al.* [56] have also reported 52.1% and 31.0% from Kenya and Uganda respectively. Moreover, 14.5% prevalence of *Leucocytozoon* spp. has been reported from Bangladesh [38], 6.8% reported from Garut [57] and 24.4% from Pakistan [39]. These findings specify the occurrence of *Leucocytozoon* species and suitable vectors for the transmission of this haemoparasite amongst village chickens in these parts of the world. However, the reasons for variation in the prevalent rates from various studies might be attributed to variation in ecologic and climatic factors, as well as dissimilarity in the management and husbandry systems in the rearing of village chickens.

The result of the present study also considered the association between prevalence of avian malaria parasites and sex of village chickens in the study area. This result of this study revealed statistical significant (p-value = 0.0003) association between prevalence of *Plasmodium* spp. and sex of chickens in the study area. The prevalence was found to be higher in male (9.6%) compared to the female (3.4%) chickens. This finding might be attributed to the facts that the male (cocks) chickens customarily have larger comb and wattle compared to the females (hen). The fact that the comb is richly vascularized and easily accessed by blood sucking arthropods including the mosquitoes, this might enhance ease transmission of the parasite during blood meal by infected arthropods. This finding agrees with that of Valkiūnas [9], Valkiūnas *et al.* [58], Opara *et al.* [42] and Hasan *et al.* [49] who have reported predominant prevalence of avian *Plasmodium* species in cocks than in the hens of scavenging chickens. Moreover, in an experimental infection involving exposure of both sexes of chickens to *Plasmodium* infected arthropods, the cocks were reported to be more infected with avian malaria, showing all evidence of the disease and corresponding clinical signs [58,59]. Several researches have also shown high prevalence of avian malaria due to *Plasmodium* species in male birds compared to their female counterparts [19,59, 60-63]. However, the result of this present study is inconsistent with the findings of Etisa *et al.* [46] who have reported higher prevalent of *Plasmodium* infections in hens compared to the cocks, and Igbokwe *et al.* [20] who have reported equal prevalent rates amongst both sexes of village chickens.

The result of the present study revealed higher

prevalence of *Haemoproteus* species in male (4.0%) compared to the female (1.1%) chickens; and association between prevalence and sex was statistically significant (*p*-value = 0.0092) at 95% confidence interval. The findings of this present study agrees with Islam *et al*. [55] who have also reported higher prevalence of *Haemoproteus* species cocks compared to the hens. The finding of this study was not consistence with Sabuni *et al*. [23] who reported high prevalence of *Haemoproteus* species in hens (4.2%) compared to the cocks (2.8%).

The prevalence of mixed *Plasmodium* and *Haemoproteus* species infection in village chickens from the present study also revealed higher prevalent rate in male (4.2%) compared to the female (0.8%) chickens, and the association between the prevalence rate and sex was statistically significant (*p*-value = 0.0009) at 95% confidence interval. To the best of our knowledge, this is the first reported prevalence of mixed *Plasmodium* + *Haemoproteus* species infection in village chickens in Nigeria, considering the sexes as risk factor. However, high prevalence of mixed infection in cocks compared to hens might also be attributed to more prominent blood sucking sites on the cocks compared to hens, even though, the hens also have comb and wattle, but are smaller compared to the cocks. The blood sucking arthropod vectors usually prefers more accessible and less feathered parts of the host birds during blood meal [64].

The result of the present study only detected *Leucocytozoon* species in male (0.8%) and the parasite was not detected in female (0.0%) chickens. The finding of the present study agrees with Sabuni *et al*. [23] who have reported higher prevalence rate of *Leucocytozoon* species in cocks (54.2%) compared to the hens (50.0%) village chickens. However, the result of the present study is inconsistent with Etisa *et al*. [46] and Hasan *et al*. [49] who have reported higher prevalence of *Leucocytozoon* species in hens compared to the cocks of village chickens. The susceptibility of village chickens to *Leucocytozoonosis* is determined by the abundance of the vector responsible for the transmission of the parasite among host [46]. Few studies that have reported the prevalence of *Leucocytozoon* species in village chickens did not revealed it's the association between its prevalence and sex of chickens [36,38,39].

The findings of the present study revealed higher prevalence of *Plasmodium* species in adult (10.2%) compared to the grower (2.8%) chickens; the association between the prevalent rates and age group was statistically significant (*p*-value = 0.0050) at 95% confidence interval. This finding concurs with Etisa *et al*. [46] who have also reported high prevalence of *Plasmodium* species in adult (20.1%) compared to the grower (10.1%) village chickens. Moreover, *Plasmodium* species have been reported to be highly pathogenic in adult chickens compare to younger ones, and mortality rates ranges from 30 - 80% [65]. However, Hasan *et al*. [49] have reported high prevalence of *Plasmodium* species amongst younger (2.9%) compared to the adult (1.2%) domesticated chickens. However, Sabuni *et al*. [23] reported that adult and grower birds shares equal chances of getting infection where exposed to *Plasmodium* infection where suitable vectors are abundant. While in Zimbabwe, Permin *et al*. [45] reported that the differences in prevalence of *Plasmodium* species were not significantly different between the bird's ages (young and adult). The present study represents the first reports on comparison of occurrences of *Plasmodium* species between village chickens' ages in Nigeria. The few reports from some parts of Africa revealed that differences in prevalence of *Plasmodium* species among ages of chickens is most likely connected to abundance of arthropod vectors and variations in exposure of host to infected vectors [45].

The prevalence of *Haemoproteus* species in village chickens from the present study revealed higher prevalent rate in adult (4.3%) compared to the grower (0.8%) chickens; the association between prevalence and age group was statistically significant (*p*-value = 0.0132) at 95% confidence interval. This finding is consistent with that of Islam *et al*. [55] who have reported high prevalence of *Haemoproteus* species in adult compare to the grower chickens. However, Sabuni *et al*. [23] have reported 4.2% prevalent rate of *Haemoproteus* specie in growers and did not detect the parasite in adult village chicken. Moreover, the finding of the present study coincides with Samani *et al*. [66] and Momin *et al*. [67] who in a similar study reported higher prevalence of *Haemoproteus* species in adult compared to young pigeons. The present study also represents the first reports on comparison of occurrences of *Haemoproteus* species between village chickens based on age groups in Nigeria.

The prevalence of mixed *Plasmodium* and *Haemoproteus* species infection in village chickens from the present study also revealed higher prevalent rate in adult (4.3%) compared to the grower (0.6%) chickens; and the association between the prevalent rates and age group was also statistically significant (*p*-value = 0.0035) at 95% confidence interval. The finding of the present study could not be thoroughly compared and discussed due to paucity of literature on the prevalence of mixed *Plasmodium* and *Haemoproteus* species infection in village chickens according to the age group. The present study also represents the first report of mixed *Plasmodium*

and *Haemoproteus* species infection in village chickens in Nigeria.

The findings of the present study revealed higher prevalence of *Leucocytozoon* species in adult (0.8%), but the parasite was not detected in grower (0.0%) village chickens. The finding of the present study is consistent with that of Sabuni *et al.* [23] who reported that *Leucocytozoon* species showed an increase in prevalence rate with increase in age of chicken. The finding of the present study did not concur with those of Etisa *et al.* [46] and Hasan *et al.* [49] who have reported higher prevalence of *Leucocytozoon* species in grower compared to the adult village chickens. However, Sehgal *et al.* [68] in a similar study reported that young birds are more susceptible to *Leucocytozoon* species than adults, and the most serious mortality generally occurs within the first few weeks of hatching.

The prevalence of avian malaria parasites according to season revealed significantly higher prevalence of *Plasmodium* species during the rainy (10.0%) compared to dry (3.0%) season. This finding agrees with Igbokwe *et al.* [20] and Okanga *et al.* [69] who have also reported high prevalence of *Plasmodium* species during the raining season compared to other seasons. The prevalence of *Haemoproteus* specie recorded in the present study was found to be significantly higher during the rainy season (3.6%) compared to dry (1.5%) season. The detection of this parasite might be connected with the rainy season considered as the favourable breeding season for several species of flies and other arthropod, which are capable of transmitting *Haemoproteus* specie to susceptible chickens. This agrees with the findings of Islam *et al.* [55] and Smith and Ramey [70] who have also reported high prevalence of *Haemoproteus* species in domesticated poultry species and waterfowls during the raining season. According to Adriano and Cordeiro [71], *Haemoproteus* species is reported to be transmitted by blood sucking insects including mosquitoes, biting midges (*Culicoides*), louse flies (*Hippoboscidae*) and tabanid flies (*Tabanidae*) whose population usually increases in the rainy season.

The prevalence of mixed *Plasmodium* and *Haemoproteus* species infection was also found to be higher during the rainy season (4.0%) compared to dry (0.9%) season. This finding is consistent with Smith and Ramey [70] who have reported high prevalence of mixed *Plasmodium* and *Haemoproteus* species infection in waterfowls during the raining season.

The prevalence of *Leucocytozoon* specie was only recorded during the rainy season in the present study, but the parasite was not detected in village chickens during the dry season in the study areas. Seasonal factors tend to have contrasting effects upon different vector species; seasonal variation in the prevalence of vector-borne diseases is well documented [69,72], and the finding of the present study are in accordance with findings from other studies of avian haemoparasites [73-76].

5. Conclusions

In conclusion, the present study revealed an overall prevalence of 23.8% for three (3) species of avian malaria parasites among village chickens in the study area, which included *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* species. *Plasmodium* species which have been reported worldwide as the cause of avian malaria in birds was found to be the most prevalent haemoparasites in village chickens in the study area. Prevalence of the three avian malaria parasites were significantly higher in cocks compared to hens, as well as in adults compared to the growers and their prevalence rates was also found to be higher in the rainy season compared to the dry season of the study period.

6. Recommendation

From this present study it was recommended that further researches involving constant surveillances and molecular characterization should be conducted to unveil the true species of the avian haemoparasites infecting village chickens in Nigeria as well as investigating the possible vectors transmitting these haemoparasites amongst the village chickens so as to design adequate biosecurity and control measures. It is also recommended that similar researches should be conducted to determine the prevalence of avian haemoparasites in other village poultry species, and to further understand the epidemiology of avian malaria in village chickens in the study area.

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Conflict of Interest

The authors declare that they have no competing interests.

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ARTICLE

Investigation of Two Precipitation Methods for Extracting Immunoglobulin Y (IgY) from Egg Yolks

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ABSTRACT

Two groups of hens (control and immunization group) were arranged in an experimental design with an immunization schedule of 3 injections of BSA antigen. IgY antibodies were extracted from egg yolks by two precipitation processes (chloroform and polyethylene glycol precipitates) and quantified using a standard curve of protein concentration. The purification of IgY was confirmed by SDS-PAGE. Total protein extracted from egg yoks were less contaminated with yellow pigments (lutein and zeaxanthin) by using chloroform precipitate. The 2nd week post-immunization, IgY concentration increased respectively to 3903 ± 726 $\mu g.ml^{-1}$ (chloroform extraction process) and 2937 ± 294 $\mu g.ml^{-1}$ (PEG extraction process) ($P < 0.01$). After 3rd immunization, IgY level obtaining from in immunization group extracted by chloroform process (6633 ± 1166 $\mu g.ml^{-1}$) increased 2.7 times higher than that in control group (2482 ± 414 $\mu g.ml^{-1}$). Whereas IgY concentrations obtained from PEG extraction process were not significantly different between the experimental group and control group. Chloroform and PEG precipitation methods had the same protein profile on the SDS-PAGE. IgY antibody was identified by the presence of bands corresponding with IgY heavy chain (67-70 kDa) and IgY light chain (25 kDa) for both precipitation processes.

1. Introduction

To deal with challenges from many diseases in livestock husbandry and antibiotic resistance, the research focus approaches the therapeutic alternatives in terms of animal welfare and suitable environment as well as reduction of undesirable effects on animals. Besides various solutions, the protective effect of egg yolks obtaining from hens immunized by specific antigen has been applied in many studies.

The collection of IgY antibodies from egg yolk is more advantageous in comparison to the collection of

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antisera from mammals because IgY antibodies can be extracted out of animals (via egg yolk thereby ensuring the animal welfare and having a lower cost compared with antisera). The antibodies extracting from chicken eggs is higher level of purity by avoiding contamination of other antibodies such as IgA or IgM generated by the humoral immune responses [13,22]. In addition, the IgY concentration extracted from egg yolks increases significantly overtime throughout the life - span of hens [3]. Successful extraction and purification of IgY from egg yolks will provide the basis for producing specific types of IgY antibodies against various antigens, which have been clinically tested as functional foods and protective agents for the body, etc. [10,25]. Powered whole eggs or yolks have been used in veterinary medicine of immunoglobulin Y (IgY) source for the treatment of enteric diseases [2]. The activity of IgY against gastrointestinal pathogens such as Enterotoxigenic *Escherichia coli* in vitro [16] and *Salmonella* Typhimurium on mice [15] showed the specific binding of these IgY to pathogens thereby reducing the mortality of infected animals. The efficacy of IgY against *Clostridium difficile* spore in the gastrointestinal tract of mice was also recorded [18]. In addition, the application of IgY in practice to replace antibiotics in prevention and treatment promises to reduce antibiotic resistance in animal husbandry [21]. Furthermore, other studies showed that the use of yolk antibodies to treat against group A Rotavirus in cattle [26], porcine epidemical diarrhea virus [14] significantly reduced the severity and mortality of infected animals. Moreover, IgY antibodies are also applied in the techniques to diagnose infections of viruses, bacteria, parasites such as ELISA, immunochromatography, Western blot [27,29].

The extraction and purification of IgY antibodies from egg yolk are increasingly attracting the interest of the scientific community as demonstrated by the significant growth of interest regarding IgY in literature. However, the purification and the quantity of IgY antibodies collected after extraction varies greatly depending on the method of extraction. Therefore, standardizing the extraction procedure is a fundamental step in IgY application [8]. The most important of purifying IgY is the separation of proteins (levitins) from lipoproteins (lipovitellins) and the rest of the yolk lipids using various chemical substances [5]. The york lipids must be removed, leaving the IgY antibody in the hydrophilic protein fraction (HPF). The different components of HPF include IgY, α-and β-livetin, low density lipoproteins, and albumin [9,12]. The precipitation of lipid from HPF depends on type of precipitating agent applied [6].

The purpose of this study was to evaluate the production of IgY antibodies in hens and to determine IgY extraction processes based on two types of precipitating agent (chloroform and PEG).

2. Materials and Methods

Immunization schedule and egg collection

A total of six hens (19 weeks old, non-clinical signs of disease) purchased from a healthy commercial layer farm were raised in cages with temperature control (25 – 32 °C) and fed with a commercial feed. Hens were divided into experimental group and control group, each group included 3 hens which were raised in individual cage and labeled as A1-A3 (immunization group) and C1-C3 (control group). Bovine serum albumin (BSA, 20 $mg.ml^{-1}$, AJ642, Bio Basic Inc., Canada) was used as antigen to immunize hens. BSA was diluted in PBS 1X solution and emulsified with an equal volume of complete Freund's adjuvant (F5881, Sigma-Aldrich Inc., USA) to obtain the final solution having 1 $mg.ml^{-1}$ BSA and then stored at 4 °C.

The immunization process based on the description of Sunwoo et al. (1996) [24]. Briefly, all of hens of the immunization group were injected with the immunization schedule of three times. In the first immunization, hens of immunization group were injected with 1 mg BSA into chest muscles at 4 sites (IM, 0.25 mg/site). Hens were treated with the same antigen dose in second immunization (2 weeks post 1^{st} immunization) and last immunization (3 weeks post 1^{st} immunization). Hens of the control group were injected with sterile normal saline (NaCl 0.9%) in the same schedule as the immunization group. Each hen's eggs were collected daily from 1 week before the first immunization to 6 weeks post 1^{st} immunization. Eggs were collected and marked according to the order of egg collection date and stored at 4 °C for IgY extraction.

IgY extraction and precipitation from egg yolks

A total of 126 eggs (3 eggs /hen/week, from 1 week before the 1^{st} immunization to 6 weeks post 1^{st} immunization) were assessed to IgY extraction. Eggs were broken carefully to discard egg white, then egg yolks were rolled gently on tissue paper to remove egg white residues to obtain egg yolks. The yolk membranes were punctured by pipette tip and egg yolks were transferred to a graduated cylinder and recorded egg yolk volume. The IgY extraction process was carried out using two separate precipitating agents, i.e., chloroform and polyethylene glycol (PEG).

For IgY extraction using chloroform, 15 ml egg yolk was mixed vigorously with 25 ml PBS 1X solution, (PD0100, Bio Basic Inc., Canada), then 20 ml chloroform

(67-66-3, VN-Chemsol Co., Ltd., Vietnam) was added and mixed for 30 seconds to obtain a homogenous mixture. The mixture was continuously centrifuged at 2,000 rpm for 30 minutes. The supernatant was filtered through filter paper and decanted to another tube for precipitation of IgY.

For IgY extraction using PEG, 15 ml egg yolk was mixed vigorously with 7.5 ml PBS 1X solution and then 7.5 ml PEG 14% solution prepared by diluting PEG 6000 powder (Polyethylen glycol 6000, PB0432, Bio Basic Inc., Canada) with PBS 1X solution was added and mixed for 30 seconds to obtain a homogenous mixture. The final mixture was centrifuged at 4 °C for 15 minutes (Hettich Rotina 35R, 9,990 × g). The supernatant was filtered through filter paper and decanted to another tube for precipitation of IgY.

The supernatant containing IgY antibodies from both extraction processes was precipitated by adding an equal volume of PEG 24% solution, then mixed for 30 seconds and centrifuged at 4 °C for 15 minutes at high speed (Hettich Rotina 35R). The supernatant was discarded, and the pellet was dissolved again in PBS 1X solution to reach the original volume of egg yolk, an equal volume of PEG 24% solution was added and then centrifuged (10,000 × g at 4 °C for 15 minutes) for the second precipitation. After centrifugation, the supernatant was discarded and the pellet was centrifuged two more times to remove residues of PEG, then dissolved in 10 ml PBS 1X and stored at 4 °C.

IgY quantification

The IgY concentration of extracting samples was determined by using protein quantification KIT-rapid (51254, Sigma-Aldrich Inc., USA). The standard curve of protein concentration was established as the manufacturer's procedure and used to determine the IgY concentration in extract samples.

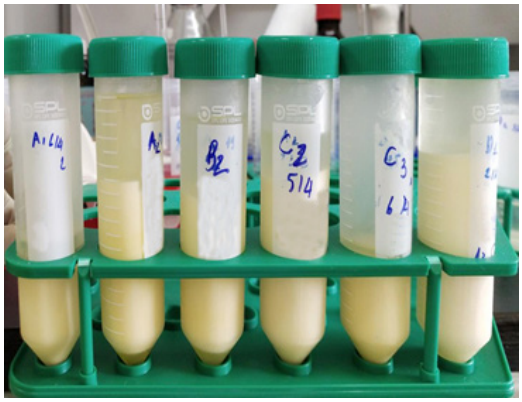
IgY confirmation by SDS-PAGE

Extraction of chicken IgY samples were checked by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. Briefly, 8% separating gel was prepared vertically in a chamber and then 5% stacking gel was added on the top. One ml of IgY extraction sample (stored in PBS 1X) was centrifuged at 12,000 rpm for 10 minutes and then discarded the supernatant to obtain the pellet of protein. SDS 10% and loading dye (20 µl and 10 µl, respectively) were added to the pellet, mixed, and then incubated at 95˚C for 10 minutes. After that, Molecular weight marker (P7703S, BioLabs Inc., USA) and samples were loaded into wells of stacking gel and the whole gel was put in a vertical electrophoresis system (Apelex Mini-Vertigel 2 PC, Cat. No. 400800, France). The gel was run under the electric field of 50 volts for 30 minutes and then 80 volts for 120 minutes. When the electrophoresis procedure was finished, the gel was treated with isopropanol for 30 minutes to fix proteins then stained with Coomassie for 3 hours and destained by 10% acetic acid until the bands on the gel were clear to observe. In total, 18 and 14 samples extracting by chloroform and PEG methods, respectively, were checked by SDS-PAGE.

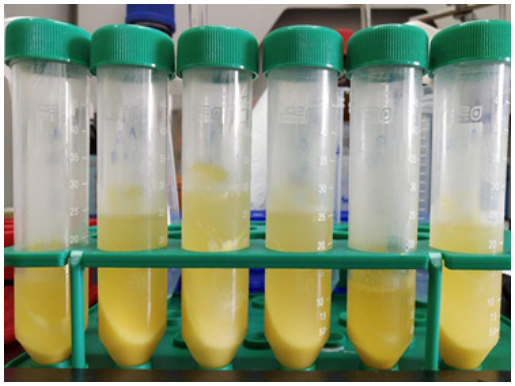
3. Results

Extraction and precipitation process

The total of 120 eggs was qualified to extract IgY using either chloroform or PEG precipitate agent. In this study, the supernatant (after lipid removal) obtained from chloroform extraction process was more homogenous opaque than that from PEG extraction process (Figure 1A and 1B). Extract proteins form egg yoks were less contaminated with yellow pigments (lutein and zeaxanthin) by using chloroform precipitation (Figure 2A and 2B).

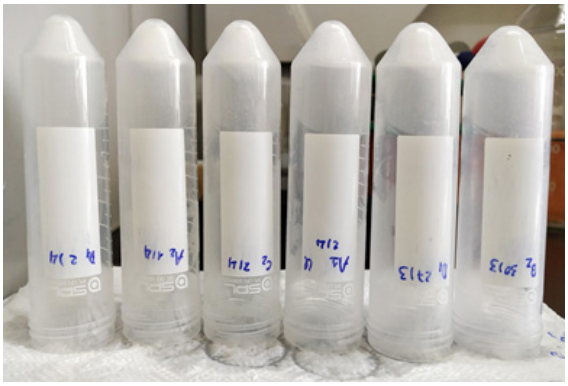


A

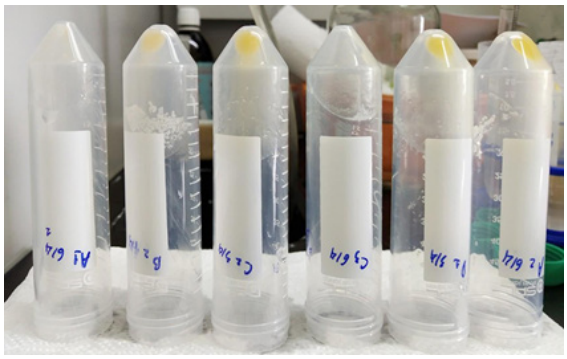


B

Figure 1. Lipid removal by (A) chloroform precipitation process and (B) PEG precipitation process



A



B

Figure 2. Protein obtained after lipid removal at 1st precipitation with (A) chloroform precipitate and (B) PEG precipitate

IgY concentration

IgY concentration collected from chloroform extraction process ranged from 536 – 7805 µg.ml^{-1} (n=62), and it ranged from 412 – 5874 µg.ml^{-1} (n=58) in PEG extraction process. One week before immunization, IgY concentration obtained from both extraction methods ranged from 592 µg.ml^{-1} to 883 µg.ml^{-1} ($P > 0.05$). The 1st week post immunization, IgY concentration was no significant difference between immunization group and control group. Otherwise, 2nd week post-immunization, IgY concentration increased respectively to 3903 ± 726 µg.ml^{-1} (chloroform extraction process) and 2937 ± 294 µg.ml^{-1} (PEG extraction process) ($P < 0.01$). After 3rd immunization, IgY level obtaining from in immunization group extracted by chloroform process (6633 ± 1166 µg.ml^{-1}) increased 2.7 times higher than in control group (2482 ± 414 µg.ml^{-1}) (Figure 3).

Meanwhile, our results showed that the IgY concentration obtained from PEG extraction process were not significantly different between the experimental group and control group (Figure 4).

Identification of IgY by using SDS-PAGE

Total protein obtained from extraction process was separated on polyacrylamide gel and IgY was detected using SDS-PAGE (Figure 5) under reducing conditions. SDS-PAGE results demonstrated that IgY extracted with

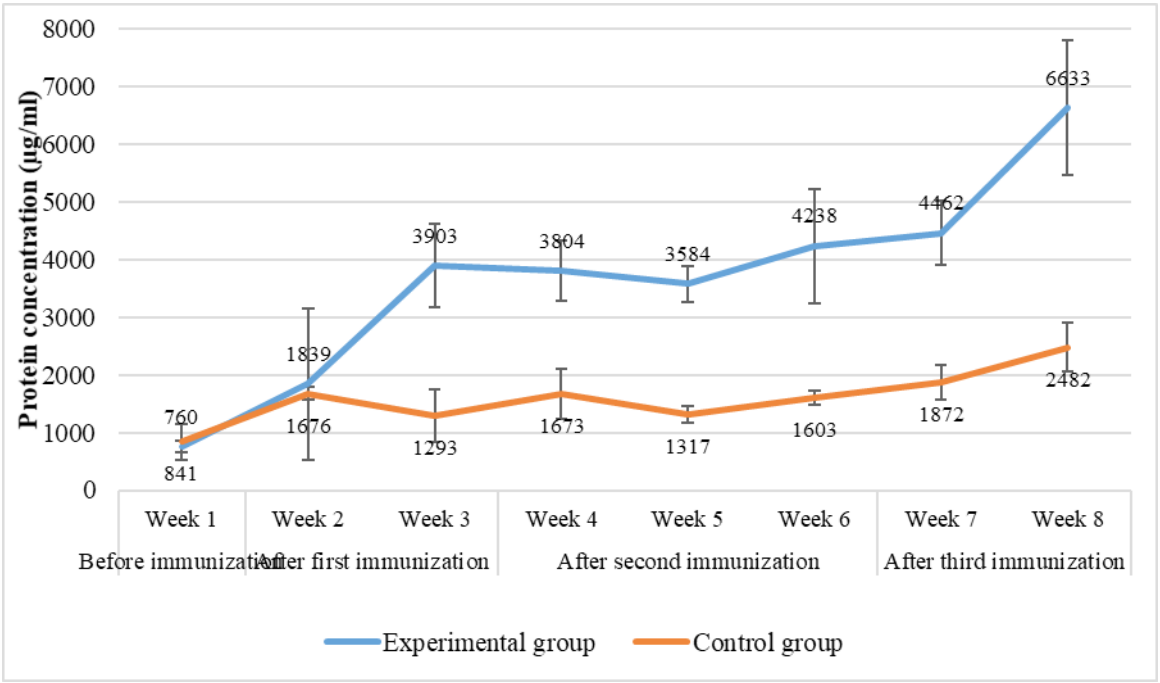


Figure 3. IgY concentration (µg.ml^{-1}) from egg yolks with chloroform precipitation method

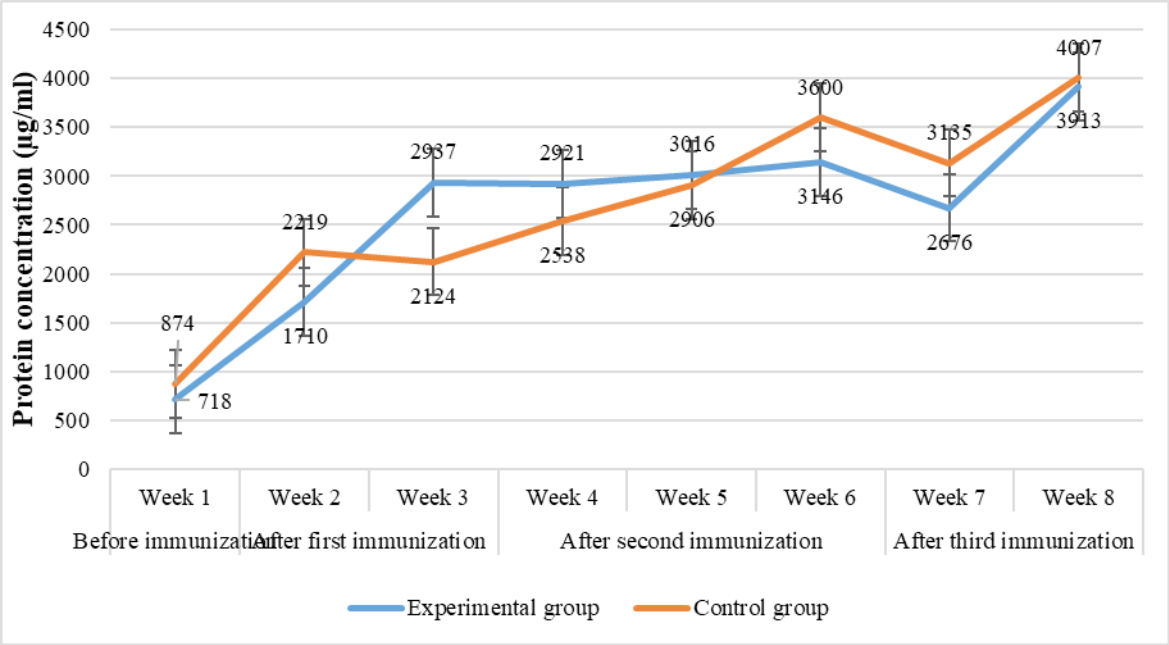


Figure 4. IgY concentration (µg.ml^{-1}) from egg yolks with PEG precipitation method

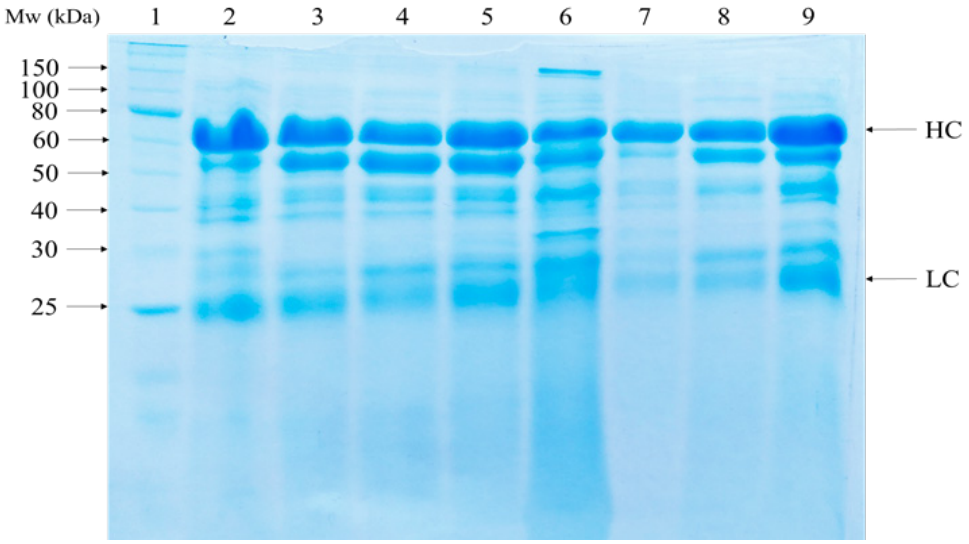


Figure 5. Identification of IgY by SDS PAGE. Lane 1: Molecular weight marker; Lane 2-7: Profile of IgY with PEG precipitation method; Lane 8-9: Profile of IgY with chloroform precipitation method; HC: heavy chain (67-70 kDa), LC: light chain (25 kDa).

PEG and chloroform containing two distinctive major protein bands which included the heavy (67-70 kDa) and light (25 kDa) chains of IgY antibodies. However, IgY purified with chloroform precipitation method contained 1 major protein band and 3 minor protein bands, also IgY purified by PEG precipitation method contained 1 major protein band and 3 minor bands.

4. Discussion

Our results showed that it is possible to generate IgY antibodies from chicken eggs using a specific antigen inducing an immune response by booster antigen injection. Therefore, the production of polyclonal antibodies through the chicken immunization showed an excellent alternative that collect antibodies with a large and qualified amount from simple methods without the need for invasive techniques [4].

Both chloroform and PEG precipitates were evaluated in some studies but their results fluctuating according to many factors. The selection of IgY crude extraction

method based on the recovery and purity of IgY levels obtained from extraction process. The selectivity of precipitation directly depends on the type of precipitating agent applied. However, several precipitation steps using organic solvents are necessary to obtain a purified protein [5]. In this experiment, IgY concentration was higher in samples purified by chloroform precipitate than by PEG precipitate. This was in accordance to another study, in which, the chloroform precipitation method reached the highest protein concentration compared to Dextran-PEG, charcoal-PEG, and PEG extraction process. Therefore, the yield of protein obtained was also highest with chloroform method (61%) than various methods such as dextran-PEG, charcoal-PEG and PEG method (8%, 50% and 26% respective) [3].

PEG was first described by Polson et al. (1980) [20] for IgY extraction from egg yolks, and the PEG method can be seemed as the standard technique [22] (Schade et al., 2001). However, this method results in a low titer of IgY and could be explained because of the loss of more than half IgY in lipid removal step by 3.5% PEG [19]. Therefore, organic solvents as chloroform were used to increasing lipid removal step from egg yolk. Previous study showed the higher content of IgY obtaining from chloroform precipitating process comparing to PEG precipitating process [3]. However, the IgY obtained by chloroform method couldn't be used to produce diagnostic reagents due to inadequate residue. Instead, these IgY is suitable for measuring antibody titers after infection or inoculation [22]. However, the precipitation by using polyethylene glycol offered a cheap and easy methodology and could be used in laboratory practice [6]. In another study, the PEG precipitation method gave several advantages such as simple, requires few steps, and yield is high by optimizing PEG precipitation method, the yield of IgY could reach 30.904 mg/mL, 6.82 mg/g egg and 392.030 mg/egg in the studied lines of chickens. Therefore, PEG precipitation method might be applied for large-scale production, and it opens the venues of using IgY in human and veterinary medicine for therapeutic and prophylactic purposes [11].

IgY purified with PEG method resulted in a significantly low total protein content compared with chloroform purification method, this result agrees with Akita and Nakai (1993) [1]. Our results are in accordance with the conclusion from Al-Edany (2013) [3] who found that the higher concentration of IgY obtained from chloroform precipitation method compared to PEG precipitation. The chloroform seems more effective than PEG in the precipitation step for the extraction of IgY from egg yolks. However, with the same schedule of immunization and egg collection, the IgY concentration by both chloroform and PEG methods are higher than our results (12.8 ± 0.25 mg.ml^{-1} and 4.4 ± 0.36 mg.ml^{-1}, respectively) [3]. This indicates that IgY levels could be influenced by the interaction of several factors (such as chicken breeds, hen ages, housing temperature, feed). The result of another study showed the possibility of generating large quantities of highly pure IgY from chicken eggs and concluded the large differences in yield of IgY production between the two studied breeds [4].

SDS-PAGE results showed that both extraction methods had the same purification ability of IgY from egg yolks. SDS-PAGE analysis of IgY purified with chloroform extraction method appears to confirm previous observation by Bizhanov *et al*. (2004) [5] who reported that the IgY extracted with chloroform is contaminated with 20% unwanted non-sense proteins.

The protein profile seems more purified due to the limitation of contaminant bands presented in our study (1 major band and 3 minor bands) in comparison with the result of Al-Edany (2013) [3] (4 major protein bands and 3 minor bands with PEG method; 4 major protein bands and 5 minor bands with chloroform method). Otherwise, by optimizing the purification process of IgY (increasing the centrifugal speed at 13,000 x g and completing with the dialysis step, Pauly *et al*. (2011) [17] showed IgY protein profile were more purified due to SDS-PAGE result presented only one minor contaminant band.

5. Conclusions

In conclusion, IgY antibodies were successfully purified from egg yolks by both chloroform and PEG precipitation methods, which were identified in IgY profile as using SDS-PAGE. However, IgY concentration extracted with chloroform precipitation method resulted in a significantly higher total protein content compared with PEG purification method. Isolated IgY with chloroform protocol might be evaluated for large-scale production by optimizing the purification step to obtain higher amounts of IgY antibodies and avoid contaminant proteins.

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ARTICLE

Economic Impacts of Clinical and Sub Clinical Mastitis on Dairy Farms

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ABSTRACT

Studies have reported on the economic impacts of clinical and subclinical mastitis on dairy farms. Bovine mastitis is a disorder that affects dairy farms and has a major economic impact. Most of the economic losses are the result of mastitis. Mastitis is an invasive infection that is among the most numerous and highly complicated infections in the dairy sector. Mastitis is one of the most expensive diseases in terms of production losses among animal diseases. Mastitis reduces milk production, changes milk composition, and shortens the productive life of infected cows. Farmers must concentrate on avoiding mastitis infection whilst putting in place and following a mastitis control programed. Bovine mastitis, the most significant disease of dairy herds, has huge effects on farm economics. Mastitis losses are due to reduced milk production, the cost of treatments, and culling. Major factors related to low milk yield could be low genetic potential as well as poor nutritional and managerial approaches. Most of the losses are related to somatic cell count (SCC), which is characterised by an increase in the percentage of milk. Culling costs are the costs of rearing or buying a replacement animal, mostly heifers. Overhead impacts include the replacement animals' lower milk supply effectiveness. The expense of replacing animals prematurely due to mastitis is one of the most significant areas of economic loss.

1. Introduction

Mastitis is perhaps the most common and expensive infectious disease that plagues dairy cows. Mastitis has a substantial influence on milk output, milk quality, and the herd's management. Bovine mastitis is marked by a prolonged and inflammatory response of the mammary gland to either physical trauma or infections caused by microorganisms. It's a potentially deadly mammary gland

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infection that's most prevalent in dairy cows all around the world. Pakistan ranks in a significant position among the major dairying countries around the globe. Major factors related to low milk yield could be low genetic potential as well as poor nutritional and managerial approaches. These factors affect the milk producing organs [1]. [2] checked the incidence of mastitis in different species. They found the occurrence of *S. aureus* to be 61.64%. The maximum resistance (58.6%) against penicillin was observed. It was concluded that resistance may be transferred to children by consuming affected milk. Among the various threats faced by dairy animals, mastitis are one of the major livestock problems in Pakistan [3]. Mastitis causes economic losses that include not only the cost of antibiotics and management practices, but also milk loss, premature culling, mortality, and infected milk discarding [4]. Mastitis is generally categorized on the basis of duration (acute or chronic) and on the basis of the appearance of symptoms (subclinical and clinical). Subclinical mastitis infections don't cause any visible changes in milk or udder appearance, making it difficult to detect. Clinical mastitis is inflammation of the mammary gland showing all signs of inflammation in teats and udder as well as in milk, while in subclinical mastitis there is inflammation but no signs are evident in teats and udder. Subclinical mastitis triumphs over clinical form in terms of economic losses. Clinical mastitis, on the other hand, is a major concern in terms of animal welfare [5].

According to the findings, the [6] somatic cell count (SCC) criterion for milk quality might be a significant part of a control strategy. In all, 396 quarters were taken from nursing cross-bred cows (Holstein & Zebu). Intra-mammary infection was seen in 56% of these quarters. Mastitis is an epidemic illness that is among the most prevalent and significant infections in the milk industry. Mastitis has an effect on food quality, direct and indirect, related to technical and hygienic characteristics and fundamental milk composition [7]. Reported mastitis is still the most expensive medical and financial problem in the milk production industry. Dairy farmers should priorities mastitis prevention while developing and adhering to a mastitis control programmer. Farmers may wait until mastitis occurs before taking steps to begin fixing the problem. The farmer's complete understanding and precise classification of mastitis-causing chemicals are critical to success.. New therapeutic approaches which do not rely on antibiotics may be required. The majority of studies on mastitis' economic impact have been undertaken in wealthy countries. [8]. Mastitis losses are compensated for by reduced milk production, culling, and treatment expenses, which account for 8%, 14%, and 78%, 8%, respectively. Mastitis' economic impact varies and should be calculated at the farm or herd level because it is influenced by local, regional, epidemiological, managerial, and economic factors [9]. Subclinical mastitis (SCM) is characterized as a rise in the concentration of somatic cell count (SCC) in milk, which many farmers underestimate due to the lack of evident abnormalities in milk. Due to the incidence of both subclinical and clinical mastitis on the farm, disease management results in increased disease losses [10].

Clinical mastitis has an extremely low death rate on average, although predicted culling happens more commonly after clinical and subclinical mastitis [11]. All dairy farms incur losses resulting from environmental mastitis, according to [12]. The value of antibiotics in controlling environmental mastitis is greatly reduced compared to their value in controlling contagious pathogens. Mastitis is often regarded as one of the most prevalent infectious diseases causing economic damage in the dairy sector worldwide [9]. Mastitis has severe consequences due to the disease's chronic condition, large economic losses that occur every year, and a dramatic fall in milk output [8] in the mammary gland. Cow performance was assessed in terms of milk output, milk composition, and mammary inflammation levels. Reported [13] that mastitis has been observed to have a direct impact on the technical attributes and sanitary quality of milk, as well as an indirect impact on its inherent qualities. Milk is one of the most essential nutritional foundations for the vast majority of a populace. Milk output has increased as a result of natural screening, and also enhanced cow feed and care, according to [14]. Poor udder fitness, particularly owing to mastitis, is among the most significant impediments to high milk supply. About 60% to 70% of the anti-microbials used on dairy-farms are used to prevent and cure mastitis. Mastitis sig Mastitis has been shown to be the maximum portent monetary apprehension in the dairy sector, as well as a source of animal welfare issues. The California Mastitis Test was used to assess the amount of inflammationificantly reduces milk supply and farm earnings. Mastitis has the potential to endanger public health since it can spread zoonoses and illnesses caused by dietary poisons [15]. As a result of the high likelihood of infection with germs from the cattle prairie, milking equipment, and direct intake of raw milk is not suggested. As a result, milk pasteurization is required to ensure its safety and to extend its shelf life [15].

Sub-clinical mastitis in dairy buffaloes in four districts (Sialkok, Lahore, Narowaal, and Okara) of the province of Punjab, Pakistan. A total of 600 animals living under different managemental settings were screened for

subclinical mastitis. The overall recorded prevalence of subclinical mastitis was 44%. Animals which were kept in backyards showed the highest (58%) prevalence, followed by small farms in peri-urban areas (42%). Whereas in the well-organized farms with better management, the lowest prevalence (32%) was recorded. Climate and breeding at different heights significantly influence the prevalence of clinical mastitis. Local breeds (Zebu cattle) were found to be resistant to mastitis, whereas exotic breeds (Belfast cattle) showed a higher prevalence. The rate of clinical mastitis cases was 41.17% during the summer and after the monsoon season. According to [11], clinical and subclinical mastitis is by far the most common fabrication illness in dairy herds worldwide.Milk supply and content can be influenced by moderate to severe brief despondency and, in the absence of a treatment, a lengthy impact. Changes in milk composition may be practically overlooked in economic considerations due to the withdrawal interval following medication [11,8]. The major parts of the economic effects of mastitis are the decline in dairy production based on the clinical and subclinical cases, milk decommissioning, the expense of medications used for the treatment of medical instances, the labor costs associated with the treatment of quantifiable cases, and the decrease in milk sales [18]. A reduction in milk output has been identified as the component with the largest economic impact on the overall cost of mastitis. Changes in milk composition may be ignored in economic estimates due to the time it takes to recover following therapy [18].

2. Milk Production Falls

Mastitis losses are due to reduced milk production, cost of treatments, and culling.Milk supply is significantly reduced in both clinical and subclinical mastitis. Clinical mastitis has been estimated to have caused economic loss [19]. Subclinical mastitis output losses are frequently assumed to be a straight log-linear connection between SCC and test-day data [20]. However, even after complete recovery from subclinical mastitis, milk supply does not improve. According to study, an estimated incidence of mastitis has an influence on the milk supply of a dairy cow, which produces 7000 kg of milk every lactation. Mastitis, both clinical and subclinical, causes a decrease in milk production. According to the National Mastitis Council, annual losses owing to reduced output plus preventative and control expenditures in the United States surpass USD 2 billion. Both clinical and subclinical mastitis impact milk output. Even after complete recuperation, milk production does not improve, therefore the economic loss remains significant. Despite the fact that antibiotic therapy prevented preclinical mastitis from developing into clinical mastitis [21].

According to [22] the rate of the mastitis and its impact on milk yield. They discovered rate of mastitis in first, second and third equality 19.94, 33.74 and 40.74%, separately. Milk misfortunes between 0.76 to 4.56 kg/d. Complete milk misfortune was 600.87 kg for steers that progressed toward becoming mastitis in initial a month and a half of lactation. Others were identified by crowds people looking at bovines whose electrical conductivity of milk expanded by >15% when contrasted and their past multi day moving normal and had a simultaneous reduction in milk yield. It has been suggested [23] that mastitis makes tremendous money related to hardships farmers. They performed starter to recognize the microorganisms responsible for mastitis in dairy. California Mastitis Test was used to screen the subclinical mastitis they found 313(45.82%) positive. Directed an examination [24] to discover the prevalence of subclinical mastitis in primiparous Simmental meat dairy cattle. To discover subclinical mastitis milk tests were gathered aseptically from each quarter. The 32% cows and 18% quarters were discovered positive for subclinical disease. Throughout the world, diseases of mammary gland like mastitis in small and large animals (ruminants) that cause significant loss to the dairy sector specially in milking animals. To counter act this problem by evaluating main organisms that may lead to intra-mammary infection, environment of the animal, somatic cell count, milk quality and milk yield that help to control to the incidence of infection [25] determined that mastitis causes huge loss in milking animals that lead to decline in milk production and affected milk quality. They collected a 6522 milk samples in the period of 52 months from the milking animals (buffalo, cattle, sheep and goat). The screening of milk samples was done by WST and SFMT at sub-clinical mastitis and overall prevalence was 23.18%.

3. Major Economic Problem

Bovine mastitis is a disease that primarily affects dairy farms and has a major economic impact. Mastitis is among the major economic difficulties in dairy cattle production, according to [26]. Even though there has been a general decrease in the incidence of mastitis in recent times, high incidence rates of 25-45 percent are still being revealed. Mastitis is a multi - factorial disease in which the environment, pathogens, and host (cow) all interact [27]. Bovine mastitis, an inflammation of the mammary gland, is the most common disease in dairy cattle, having caused economic costs due to decreased milk production and low milk quality. The etiological agents include a wide

range of gram-positive and gram-negative bacteria which can be highly infectious (e.g., *Staphylococcus aureus, Streptococcus agalactiae,* Mycoplasma spp.) or ecologic in nature (e.g., *Escherichia coli, Enterococcus spp*., coagulase-negative *Staphylococcus, Streptococcus uberis*). Acute mastitis is among the most infectious ailments in wide dairy cows, as [28]. Mastitis also can end up causing an inspirational dispute in a cow's behavior priority areas. To investigate this, we observed the behavior of six cows following acute mastitis induction..Cattle spent that time telling lies just on initiation day than previous day, and much less duration just on side of the acute inflammatory udder quarter. During a day, cows also spent more time overall eating silage. We propose that pain in the udder supersedes the motivational state of the cows' sickness behavior.

Mastitis is the most common and accepted disease of dairy cattle globally, causing huge losses to the dairy sector under unhygienic conditions. This becomes a serious quandary in dairy cattle, with substantial financial penalties, the risk of the spread of milk-borne diseases, and primarily a decline in milk yield and impaired milk quality [29]. Mastitis is generally classified into two types: Evert and subclinical.In clinical mastitis, the basic signs of inflammation (heat, pain, swelling, redness, and loss of function) are present, whereas in sub - clinical mastitis, there is no visible sign of inflammation. SCC in milk increased in subclinical mastitis by nearly 75-80 percent worldwide [30]. Milk quality is harmed, and production declines, reducing the income of poor farmers and causing farming yield loss points to a strong to mastitis, which directly threatens safety. Subclinical mastitis has a substantially greater prevalence than clinical mastitis. Gram-negative bacteria (25 to 30%), coagulase-negative *Staphylococcus* (20%), *Staphylococcus aureus* and *Escherichia coli* (5 to 10% each), Streptococcus and Enterococcus (2 to 5%), and other species (less than 2%) are the most common pathogens that cause mastitis [31]. Mastitis costs are determined by a variety of factors, much as other illnesses. Reduced milk production, veterinary services, diagnostics, medications, abandoned milk, and labor are the most typically addressed issues. Although factor costs vary by country and area, the economic concepts that underpin them remain the same [9,8].

Culling

The culling of animals is a tough issue to assess because it is caused by other factors (excluding in the circumstance of demise for reasons other than culling). The dairy farmer makes the decision to cull. When replacement is the best option, a cow is culled. Cows with mastitis are more likely to be culled. Mastitis is perhaps the most common production illness in dairy herds worldwide [20]. Mastitis economies must be handled at the farm and are dependent on local and regional epidemiologic, managerial, and economic variables [18].

Why *S. aureus* is of major economic impact?

Dairy farmers suffer higher economic losses due to subclinical mastitis, which is a major issue among dairy cattle. This disease is one of the most common reasons of low milk production and poor milk quality, and it is also the major cause of considerable losses for dairy farmers [32]. Mastitis is one of the most expensive diseases in terms of production losses among animal diseases that affect the profitability of raising animals [33]. According to total milk production, Pakistan ranks 4 in the world, but its dairy industry isn't very well developed as animals are kept in small group [34]. Only a few species of bacteria cause mastitis among the many are predominant. A common pathogen is *Staphylococcus aureus*. Therefore, *S. aureus* mastitis in cows is hard to eliminate. Hence the need to enhance the existing technologies used to control *S. aureus* mastitis, given its economic impact, as well as food security and antibiotic consumption challenges [35]. Mastitis control is a concern all over the world, posing a serious threat to the dairy industry and complicating animal care maintenance [36]. As *S. aureus* mastitis is a transmissible illness that banquets from infected udders to healthy cows, sanitation is critical.Cleaning and drying udders prior milking, using properly designed milking machinery, dry cow treatment, culling chronically infected cows, milking sick cows in a distinct category, and developing an dynamic milk superiority programmed are all things that should be considered [34].

To avoid reintroducing bacteria in a *S. aureus*-free herd or implementing new pathogens to an infected herd, proper biosecurity measures are critical [35]. Presence of pathogens in a healthcare product does not necessarily indicate that this product was an external source of pathogens. In a cluster of Serratia outbreaks, farm-specific strains of the pathogen were identified, and the outbreaks were associated with unhygienic handling of teat-dip, resulting in contamination with Serratia, and subsequent growth [37]. By using diagnostic tests and keeping good records of cases and treatments, mastitis control can be targeted at specific pathogens to reduce the incidence of the disease. The health management program in each herd should include special consideration for the health of the udders [38].

Consequently, further refinement is necessary before it can directly identify *S aureus* in milk samples. Recently,

the LAMP assay was reported to be able to detect *S. aureus* in milk within two hours [39]. The development of rapid and sensitive methods of determining contagiousness, pathogenicity, and antibiotic resistance, as well as cost-effective tools to test for antibiotic resistance, needs to be pursued to improve treatment and control measures. It will prevent the introduction of new, highly contagious strains and improve herd biosecurity when dry cows, non-lactating heifers, and latent carriers are tested prior to purchase [35]. Mammary glands are equipped with antibacterial systems, but their effectiveness against mastitis is very limited. Vaccination against mastitis is the most common method of improving natural immunity in dairy cows. It has taken many years for vaccines against *S.aureus* and, more specifically, *S.aureus* mastitis to make it to market. In order to be effective, an ideal *S.aureus* mastitis vaccine would prevent infection, or facilitate its clearance from the mammary gland shortly after IMI, thereby preventing long-term intra mammary infections that serve as reservoirs for herd-mates' infections [35]. The diet plays a crucial role in the resistance to disease, and some trace substances and vitamins are predisposing factors for mastitis, including selenium, copper, zinc and vitamin E. A decrease in vitamin A, E, and Zn concentrations at calving could negatively impact the immune system of the cow, following calving [34]. For dairy cows with high yields, current dietary recommendations may not be sufficient to ensure optimal immune function and response around parturition [40]. A diverse approach is required to effectively combat this condition. Many knowledge gaps, including vaccine development, are impeding progress in diagnostic, therapy, and prevention [35,39].

4. Other than *S.aureus* Mastitis for Economic Impact

Mastitis causes significant economic losses to the dairy cattle industry. Mastitis usually caused by *Escherichia coli* (*E. coli*), Streptococcus agalactiae**,** *Staphylococcus aureus*, and *Klebsiella pneumonia* [41,42].

Escherichia coli (*E. coli*)

E. coli are among the most common pathogens causing ecologic mastitis. It generally targets the mammary gland all through milk yield, and if remain unattended, it can be potentially lethal. The most major reason of mortalities is acute clinical *E. coli* mastitis. This same intensity of the infectious disease, stage of milk production, energy balance, vitamin deficiency, and vaccination status all influence the treatment outcomes of E. coli mastitis [43]. Mastitis induced by *Escherichia coli (E. coli)* continues to be a potential danger to dairy cattle, affecting animal rights and causing significant economic deficit. It is still a fatal condition in dairy cattle all over the globe. It endangers mammary gland health, reduces dairy productiveness, stifles bovine expansion, raises rearing and preventative measures costs, and has a deleterious impact on animal wellbeing [44].

Klebsiella pneumonia

Although *K.* pneumoniae is typically assumed to be an ecologic representative which is mostly prevalent in and transferred through the atmosphere, it can every once in a while spread the infection cow to a better and healthier cow [45]. This is most prevalent in bedsheets, especially shavings and perlite, which serve as main water sources for this pathogen. Water and soil seem to be two other potential environments for this microbe to survive and prosper [46].

K. pneumoniae is among the known causes of mainly environmental *Klebsiella pneumoniae* mast it is in the dairy sector and it has been the subject of many studies [47]. Clinical mastitis (CM) is a situation whereby an animal exhibits physical effects of mastitis and dairy productivity and processing are also impacted [48]. While most research shows that treatment has a minimal effect. After using antibiotic to treat non-severe instances of *Klebsiellapneumoniae* associated CM, there was a substantial increase in microbiological cure.Mastitis reduces milk yield, and most cows do not restore to normal production levels after recovery [49] resulting in significant economic losses. It's also been noted that the degree of milk supply reduction varies according to the pathogen that causes the infection, with Gram negative bacteria producing a bigger decrease than Gram positive bacteria and other non-bacterial species [49].

Streptococcus agalactiae

Numerous types of observational evidence suggest that milking employees may transfer *Str. agalactiae* into bovine herds, according to [50]. *Str. agalactiae* strains isolated from udder mastitis and human infections were found to share 58 percent genetic similarity in a research, while clustering revealed that they shared 70 percent genetic similarity [47]. In animal-to-animal transferred genotypes, the aspect of self is extremely low. Young cattle during their first feeding period are much more immune to infectious causal factors, according to circumstantial data. The disease can persist inside the udder for a long period of time and be undiagnosed. These

animals serve as reservoirs for infections and spreaders of the disease [19]. Cow mastitis is by far the most common illness in dairy cattle around the world. Bacteria, such as *Streptococcus agalactiae*, are the principal etiologic agents [18]. This agent is crucial in bovine mastitis since it is highly contagious and also has a significant impact on the development of diagnostic mastitis as well as the rise in bulk milk somatic cell counts [49].

Minor mastitis pathogens

Microbes that possibly cause mastitis Secondary mastitis infections include non-haemolytic coagulase-negative staphylococci (CNS) and Micrococcus spp. It can considerably raise the leukocyte counts in milk in rare situations. They now are one of the most frequent bacteria discovered in milk cultures, particularly in herds with sufficient control of major diseases. They are part of the natural teat flora and function as opportunist pathogens, causing disease when the conditions are right [35,49] S. *epidermidis, S. hyicus, S. intermedius, S. chromogenes, S. hominis, S. warneri, and S. xylosus*are among the coagulase-negative *staphylococci. S. epidermidis and S. hyicus*are the most usually isolated bacteria from cow mastitis. *Bacillus spp., Pseudomonas spp., and Corynobacterium spp*. are among the infections that cause mastitis less frequently. *Bacillus cereus* and *Bacillus subtilis* are saprophytic bacteria detected in mix infections and mastitis induced by teat injury among Bacillus organisms [35].

Environmental pathogens

These are pathogens common in the cow's surroundings, and intra-mammary infections result from teat end exposure to these pathogens between milking. Environmental infections are responsible for 40% of cases each year [51]. On the well farms, environmental pathogens are responsible for the majority of mastitis episodes, and the majority of IMI cases caused by environmental pathogens are attributed to coliforms and environmental streptococci [51,35].

5. Mastitis Recommendation and Prevention

The majority of recommended mastitis control techniques were considered to be economically advantageous, according to [52]. It was not cost effective to use a sanitizer in the cleaning solution and have a business adjust the milking machine inflations. The SCC of a single cow was a better predictor of milk loss than the SCC of a bulk tank. Questions have been raised about cost-effectiveness and effectiveness of treatment every cattle at dry off rather than just a few. Under field conditions, a DHI survey was paired with DHI production data to determine the association among milk production, SCC, management practices, and production parameters [51,32].

Mastitis preventive and control measures include: a) public trough disinfection, b) milking operations that comply to hygienic principles, c) removal of cows with chronic mastitis, d) dry cow treatment with antibiotics, and e) treatment of mastitis [52]. The plan also includes keeping a clean and comfortable environment by managing bedding, keeping places clean and dry, ensuring sufficient ventilation, and providing feed shortly after milking [51]. Establishing udder health goals and reviewing them to priorities changes in management to meet those goals, maintaining a clean and comfortable environment through bedding management, keeping areas clean and dry, ensuring proper ventilation, and providing feed soon after milking to keep animals in standing position [53].

6. Conclusions

The economic repercussions of mastitis (clinical or subclinical) appear to be attributable to treatment, production losses, culling, changes in product quality, and the risk of additional illnesses, according to this research. Mastitis is one of the most expensive diseases in terms of production losses among animal diseases that affect the profitability of raising animals. *S. aureus* mastitis is a contagious illness that spreads from infected udders to healthy cows. Mastitis control is a concern all over the world, posing a serious threat to the dairy industry and complicating animal care maintenance. Mastitis reduces milk production, changes milk composition, shortens the productive life of infected cows, and is very costly to the dairy farmer. The associated costs can be divided among the following factors: milk production losses. Mastitis represents a most costly health and economic issue inside the dairy industry. Mastitis is by far the most expensive significant social and economic issue in the dairy sector. Farmer must concentrate on avoiding mastitis infection whilst putting in place and following a mastitis control program. Aimed at limiting antimicrobial use and implement novel ways in dairy cattle also may aid in the prevention and treatment of this disease.

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ARTICLE

Comparison of Locomotion Problems and Its Economic Impact on Cobb and Ross Broiler Strains

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ABSTRACT

The rapid weight gain and fast muscle growth due to intense genetic selection and improved nutrition for additional breast muscle in broiler commercial strains affect chickens health. In order to compare the main locomotive problems in broilers of Cobb and Ross strains, two pens from a commercial farm in Veracruz, Mexico were used. The first pen housed 16,500 males and 16,500 females of Cobb strain and the second one 16,500 males and 16,500 females of Ross strain. Chicks were checked for locomotion problems from day one until their sale. Animals with problems were recorded and necropsies were performed to identify the pathology. Out of 1406 animals with locomotive problems (2.13% of the total), 58.9% were Cobb and 41.1% Ross ($P < 0.05$). The frequency of locomotive problems was 2.51% for Cobb and 1.75% for Ross. Most common individual lesions were osteochondrosis (38.61%), inflamed joints with purulent contents (37.13%), and valgus (19.65%). Locomotive problems appeared since the first week, but its number increased as birds gained weight, particularly from the fourth week on. Problems occurred more in males than in females and in Cobb birds than in the Ross strain. Economic loss due to locomotion problems was higher for the Cobb strain.

1. Introduction

Poultry meat production has been a very dynamic industry over the last decades. Genetic enhancements and breeding have resulted in the current broiler chicken strains characterized by faster weight gain and better feed conversion. In fact, the potential for growth and body conformation of poultry are related to improved genetics, better understanding of nutrition and feeding, and overall improved management techniques that increased the efficiency and profitability of the poultry sector [1-3]. Genetic broiler lines have a high growth rate and the formation of notable muscle masses, mainly in the

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breast and thighs. A short period of growth and fattening, around 6-7 weeks, has made chicken the main base of the production of chicken meat for consumption [4]. However, the incidence of leg abnormalities in rapidly growing broilers is higher than in other broilers chickens [5]. Bone deformation is a very frequent and serious problem that affects the well-being of chickens of this type [6]. Bone abnormalities compromise the welfare of the birds and cause harm to the poultry industry due to culling, late mortality, poor performance and carcass condemnation [7]. Even though associated pathologies to this condition are known, they have s not been widely reported, and there is not much information on its presentation in commercial farms with high population densities in tropical conditions, so the objective of this study was to compare the main locomotive problems in Cobb and Ross strains broilers in Mexico and their economic impact.

2. Material and Methods

2.1 Location and Facilities

Birds used in this study were reared under similar conditions in two conventional poultry houses from the Santa Ana farm located in the town of San Antonio, Municipality of Paso del Macho, in the State of Veracruz, Mexico, were used. This is in a tropical climate and at an altitude of 250 meters. The pens are 15 m wide by 138 m long for a total of 2070 m^2. The ventilation is tunnel type, with 11 extractors, wet walls, sensors, Gasolec brooders, Cumberland feeders, lubing drinking nipples. Also, 1 kg/m^2 of rice husk was used as litter. All management was identical for the birds, regardless of their lodging or strain.

2.2 Poultry Management

The chicks received 200 g of pre-starter from 0 to 7 days of age, 1 kg of starter feed from 8 to 21 days, 2 kg of grower feed from 22 to 34 days, and 1,800 kg of 35-day finisher feed until sale. The feed used for feeding all birds of similar age was the same. Four to five days before the arrival of the chickens, the pens are cleaned and disinfected. When in the hatchery, the chicks receive a Marek + Newcastle vectorized vaccine by subcutaneous application and the Bronchitis MA5 and 491 vaccine by micro-spray. By the eighth day on the farm, Gumboro vaccine (univax plus) is given by mouth. At the 12th day, the emulsified Newcastle vaccine (subcutaneous route) + live virus Newcastle vaccine (ocular route) is applied. On the eighteenth day, the Gumboro vaccine (univax BD) is given. The stocking density was 16.03 birds/m^2.

2.3 Experimental Design

For this study, 66,000 chickens were followed throughout their fattening period. In house 1, 16,500 males and 16,500 females of the Cobb strain were included, while in house 3, 16,500 males and 16,500 females of the Ross strain were housed. From the day of arrival to departure, animals with locomotion problems were recorded and necropsies were performed. The type of problem was determined in each case and to which group (strain and sex) they corresponded to. From week 3 on, birds weight was recorded separately on weekly basis for each of the categories, i.e. strain and sex. The average body weight gain was obtained at the end of each week for each strain and sex by dividing the total broiler weight of the animal category by the number of animals. Economic impact of locomotive problems was calculated base on production by pen (kg), gross income by pen ($USD), losses due to locomotion problems (kg), economic losses due to locomotion problems ($USD), and proportion of economic losses due to locomotion problems (%). Sale price of broilers was $USD 0.81 per kilogram.

2.4 Data Analysis

The results of daily observations on the different variables were captured in Microsoft Excel and later statistical analysis was performed using Minitab v 17 to determine differences between broiler strains (Cobb and Ross) and animals sex (female and male). To compare weekly weight lost between broiler strains, t test was performed from week 2 on.

3. Results

Out of 1406 animals with locomotive problems (2.13% of the total), 58.9% were from the Cobb line and 41.1% from the Ross line ($P < 0.05$). The frequency of locomotive problems during the fattening period was 2.51% (828/33,000) for the Cobb strain and 1.75% (578/33,000) for the Ross strain. The most common individual lesions were osteochondrosis (38.61%) and inflamed joints with purulent contents (37.13%), followed by valgus (19.65%), chondrodystrophy (4.30%) and rotation of the tibia (0.30%). However, injuries more often occurred jointly, as shown in Table 1.

Locomotive problems appeared in the first week of fattening and augmented progressively as the birds gained weight, especially from the fourth week, in which the appearance of lesions increased notably, which coincides with chickens take off in weight gaining. In the fifth week, the number of cases due to swollen joints with purulent content and osteochondrosis in the Cobb strain exceeded

by far the number of cases occurring in the Ross strain. Only in the sixth week did the Cobb strain surpass the Ross in a number of cases. However, when considering the overall period, the Cobb strain had more locomotive problems than the Ross strain. Seventy-three percent of the problems occurred in the last three weeks.

In general, in the Cobb strain, locomotive problems more often occurred in males than in females (65.58% vs 34.42%, p <0.05), except in the cases of chondrodystrophy, osteochondrosis, and chondrodystrophy and osteochondrosis, in which the frequency in females was greater. In the case of the Ross strain, males had more locomotive problems than females (64.71% vs. 35.29%, p <0.05). Only for osteochondrosis, the frequency in females exceeded that of males.

Table 2 shows that most cases of locomotive problems occurred in males of the Cobb strain (543, 38.62%), followed by males of the Ross strain (374, 26.6%). In turn, females of the Cobb strain (285, 20.27%) were more affected than females of the Ross strain (204, 14.51%). Although weight and economic losses occurred during the first two weeks, they were negligible, but mounted gradually as the time passed by, but always being greater for Cobb strain birds.

Table 1. Locomotive problems identified by broiler strain per week of fattening in a commercial farm in San Antonio, Mexico.

Week	1		2		3		4		5		6		7		Tot	%
Locomotive problem/ line*	C	R	C	R	C	R	C	R	C	R	C	R	C	R		
Inflamed joints with purulent content, osteochondrosis	1	1			6	5	17	14	82	38	123	54	63	62	466	34.40
Inflamed joints with purulent contents	10		8	7	15	4	15	13	29	32	28	25	14	11	211	14.90
Osteochondrosis	1	1	6	3	3	3	1	3	18	17	27	16	22	50	171	12.53
Osteochondrosis and valgus		1	1	1	6	1	7	4	13	10	12	7	33	42	138	10.16
Valgus	5	3	4	6	7	7	8	7	15	6	19	13	22	15	137	9.56
Inflamed joints with purulent content, valgus, osteochondrosis				1	1	1	13	12	9	6	10	4	23	19	99	7.34
Inflamed joints with purulent contents and valgus		1	3	1	6	5	20	8	7	13	5	1	3	5	78	5.71
Chondrodystrophy	28	4	32	6	6		3	2	2	1	1				85	3.93
Chondrodystrophy and osteochondrosis			6	1	3	2			2						14	1.04
Rotation of the tibia		1		1			1		2		1			1	7	0.44
Total	45	12	60	27	53	28	85	63	179	123	226	120	180	205	1406	100

*C=Cobb, R= Ross

Table 2. Weekly cases of locomotive problems identified by broiler strain and sex, and associated weight lost in a commercial farm in San Antonio, Mexico.

	Cobb			Ross			Cobb	Ross
Week	Male	Female	Total	Male	Female	Total	Weight Lost, kg	
1	22	23	45	8	4	12	-	-
2	23	37	60	16	1	17	-	-
3	23	30	53	14	14	28	24.21 a	14.8 a
4	57	28	85	42	21	63	84.17 a	60.13 b
5	132	47	179	89	34	123	250.5a	163.34 b
6	167	59	226	73	47	120	383.66a	211.07 b
7	119	61	180	132	73	205	381.05a	449.11 a
Total	543	285	828	374	194	568	1,123.59a	898.45 b

Different literal per row indicates statistically significant difference (P < 0.05).

Given that chickens of the Ross strain achieved a higher production per house of 33,000 birds, as well as lower losses caused by locomotion problems, this condition had a greater economic impact on the Cobb strain (Table 3).

Table 3. Economic impact of locomotive problems identified by broiler strain in a commercial farm in San Antonio, Mexico (in USD).

Variable	Cobb	Ross
Production by pen, kg	91677.28	92344
Gross income by pen, $USD	75784.69	75328.59
Losses due to locomotion problems, kg	1,123.59	898.45
Economic losses due to locomotion problems, $USD	916.55	315.11
Proportion of economic losses due to locomotion problems, %	1.23	0.42

4. Discussion

According to Almeida Paz (2008), locomotive disturbances affect around 6% of the animals in commercial lots [8]. In a study encompassing broiler flocks of the five major UK producers, Knowles et al. (2008) found that at a mean age of 40 days, over 27.6% of birds showed poor locomotion and 3.3% were almost unable to walk [5]. Webster et al. (2013), in a nation-wide study in new Zealand, used the 6 point (0-5) gait scoring method and determined a percentage of birds with gait score 3-5 of 30.3 ± 6.77% [9]. In a review, Hartcher and Lum (2020) declared that the prevalence of birds with moderate to severe gait impairment is between 5.5 and 48.8% [10]. The current study found that the average frequency of locomotive problems was 2.13%, ranging from 1.75% in the Ross strain to 2.51% in the Cobb strain, all well below international previous reports.

Fernandes et al. (2012) pointed out that together, femoral degeneration, tibial dyschondroplasia, and angulation deviations are the main diseases associated with lameness in broiler chickens; in addition, they may or may not occur in association [11]. A study in 28 broiler flocks of chicks reared in conventional production systems in Denmark, reported as the main problems tibial dyschondroplasia, varus/valgus deformations, crooked toes, foot pad burns, and asymmetrical development of the tarsometatarsus [12]. Vitamins D, A, C, K and B, as well as calcium and phosphorus and the relationship between them are essential to bone development [13]. The deficiency or imbalance of vitamins and minerals are associated with rickets and tibial dyschondroplasia [14]. Also, insufficient intake of vitamin D leads to increased incidence of rickets and tibial dyschondroplasia [15]. The tibial dyschondroplasia is one of the most common problems of the legs, being clinically detectable in animals older than 35 days old [16]

The risks of the occurrence of leg problems are significantly influenced by body weight and sex of the chicks [12]. According to Sorensen et al. (2000), locomotion disorders are relatively less important at 28 days than later [17] also, light broilers had significantly better footpad dermatitis and gait score than heavier broilers [18]. Hence, one of the main factors responsible of leg problems in broilers is their fast growth rate which results in a high prevalence in conventional production systems and compromises the welfare of the birds [12]. In the current study, males of the Cobb strain showed more locomotion disorders than males of the Ross strain. Sterling et al. demonstrated that Cobb broilers have better growth rate with a better feed conversion ratio than the Ross strain [19]. The Cobb strain chickens acquire a great weight quickly, allowing sacrifice at a very early age; they are voracious, have a good muscular conformation especially in breast, show a nervous temperament, and are very susceptible to high temperatures. Ross broilers also have very fast growth, exceptional feed conversion and high meat yield; these chickens have been selected for their vigor, strong legs, and powerful cardiovascular system. Stringhini et al. (2003) evaluated the performance and carcass characteristics of different broiler strains in São Salvador, Goiás, Brazil. Male broilers had better productive parameters and heavier body and carcass weight than females, but there were no differences in commercial parts yield and carcass characteristics among Ross, Lohmann y Arbor Acres strains [20]. In a study that evaluated the performance of broiler strains (Cobb 500, Ross 308, and Hubbard Flex) in hot weather, the Cobb and Ross strains showed at 49 days old the best breast yield, with the Hubbard strain having the greatest drumstick yield. Regardless of strain, the males showed superior performance to that of females [21]. Total body weight of Cobb-500 and Ross-308 on the first week was 207.40±14 gram and 196.00±16 gram respectively, a significant difference of weight gain ($P<0.05$) [22]. From the previous exposure, it is evident that the appearance of locomotion injuries is exacerbated with higher growth rates. Since Cobb chickens have a higher growth rate than Ross and their body weight is higher by the end of the fattening, then the risk of locomotion problems is increased.

Arguably, reduced growth and culling of lame birds affects farm profitability impacting production costs [23]. Poor performance is a consequence since these animals cannot feed and drink correctly [24]. Also, injured carcasses condemnation in slaughterhouses is increased [25]. In fact, condemnation at *postmortem* inspection has been

associated with increasing gait [26]. The current study found low economic losses due to locomotion problems in poultry probably as a consequence of the relatively small proportion of affected animals.

Over the past 60 years, the genetic selection of broilers has focused on production traits such as growth rate and feed efficiency. Advances in nutrition and genetics led to an increase in body growth and meat deposition rate of broiler chickens causing metabolic disturbances that damaged the production system [27]. This has led to significant problems in birds such as leg disorders and cardiovascular diseases [28]. Locomotion disorders, commonly known as deformities or locomotion problems, may occur because of changes in bone and cartilaginous growth plate [29]. The prevalence of locomotion disorders and the weakness of the bones in broiler chickens have become a big concern, as they have an important impact in the audits of animal welfare, as well as on the physical and microbiological quality of the carcasses [23,28].

Nowadays, broiler rearing system is a crucial factor affecting birds comfort, welfare, health, and production efficiency [30]. Locomotion disorders are due to multiple contributing factors such as age [31], breed and strain[32], stocking density and growth rate[33], bedding material and quality [34], air quality[35], housing type [36,37], poor temperature control in incubation room or very smooth hatch trays [38], problems in transporting the chicks to the farms and stress due to climatic variations (especially heat stress) [39], poor nutrition of the breeders (minerals, vitamins and calcium) and severe feed restriction in certain phases of life [40]. Not many therapies have been tried for this problem, but increasing levels of glucosamine sulfate supplementation in the diet increased the weight gain of age broilers [41].

5. Conclusions

In a commercial broiler farm in Veracruz, Mexico an overall prevalence of 2.13% for locomotive problems was found in a population of 66,000 birds. Cobb strain chickens were more affected than Ross strain birds, as were males more than the females. Osteochondrosis, inflamed joints with purulent contents, and valgus accounted for more of 95% of the cases. Locomotive problems occurred since the first week and increased as birds gained weight, particularly from the fourth week on. Economic loss due to locomotion problems was higher for the Cobb strain, but in average accounted for 0.83 % of the gross income. Even though locomotion problems do not seem to be a big issue, it is convenient to explore venues for prevention and treatment of this condition.

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Conflict of Interest

No potential conflict of interest was reported by the authors.

Authors' Contributions

This work was carried out in collaboration among all authors. B.L. Guevara-Torres carried out the research on the field, collected the samples, and wrote the first draft of the paper. L.A. Landin-Grandvallet and A. Tirado-Madrid designed and supervised the study. J.A. Villagómez-Cortés managed the literature search, performed the statistical analysis, and wrote the manuscript´s English version. All authors reviewed and approved the final version of the paper.

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