Virome of broiler chickens in conventional production

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1. Introduction

Domestic chickens are essential to global food security, serving as a primary source of animal protein through both meat and egg production. The gastrointestinal tract of chickens harbours a complex and dynamic microbiome, comprising trillions of bacteria and viruses that profoundly influence various physiological processes, including metabolism, immunity, nutrient absorption, and overall health and productivity (KAITHAL et al., 2016; LIMA et al., 2017; WANG et al., 2024). Although considerable research has been conducted on the role of the chicken gut microbiome in metabolism, immunity, and meat production, the impact of viruses on chicken health, particularly their contribution to gastrointestinal diseases, remains an underexplored yet critical area of study (DEVANEY et al., 2016; LIMA et al., 2019). Gastrointestinal diseases in chickens are of particular concern due to their significant economic impact, causing millions in losses in the chicken meat market (KIM et al., 2020). The increasing prevalence of intensive farming facilities has exacerbated the rapid spread of viruses within flocks, leading to weakened immune systems and subsequent infections by other pathogens. Such conditions are often associated with Malabsorption Syndrome (MSS) or Runting-Stunting Syndrome (RSS) in poultry, which are characterized by poor nutrient absorption and stunted growth (de OLIVEIRA et al., 2021). These syndromes can be triggered by various factors, including viral infections, that compromise the immune system and exacerbate gastrointestinal issues, ultimately leading to significant flock losses and financial damage (KIM et al., 2020).

Recent advancements in virome identification techniques have revealed a diverse viral community in the chicken gut. Studies have identified viruses from families such as *Adenoviridae, Caliciviridae, Circoviridae* (including Chicken Anemia Virus, CAV), *Parvoviridae, Picobirnaviridae*, *Picornaviridae,* and *Reoviridae*, all of which are associated with various gastrointestinal diseases in chickens (LIMA et al., 2017; LIMA et al., 2019). For instance, while CAV primarily affects the immune system, it can also lead to increased susceptibility to gastrointestinal viral infections (POULTRYDVM, 2024). Chicken viruses exhibit diverse transmission modes. Some viruses, such as Infectious Bursal Disease Virus (IBDV) and rotaviruses, primarily spread horizontally through direct contact with infected birds or contaminated environments (MSD MANUAL, 2024a). Conversely, other viruses, such as CAV, adenoviruses, and astroviruses, can be transmitted vertically from parent to offspring through the egg, although horizontal transmission should not be disregarded (SMYTH, 2017; KUBACKI et al., 2022). Understanding these transmission routes is crucial for implementing effective biosecurity and vaccination strategies in poultry production.

Genomic sequencing has emerged as a powerful tool for comprehensively analyze the genetic makeup of viruses, enabling the development of improved diagnostics and treatment strategies (WANG et al., 2024). Novel sequencing methods and integrative platforms enhance sample preparation and provide detailed insights into the genomic diversity of the virome (BULLMAN et al., 2014; GORAICHUK et al., 2021; LIEBHART et al., 2023). These advancements allow for real-time tracking of viral mutations and the identification of pathogenic strains that impact the health of chickens in the flock. However, the application of this tool remains under development, primarily focused on diagnostic research rather than routine practical use in flock management (LIEBHART et al., 2023). This limitation arises from the precise laboratory conditions required, which demand considerable accuracy and the expertise of trained staff (LIEBHART et al., 2023). As a result, developing fast and practical methods for analyzing gut viruses is essential to effectively prevent the spread of enteric diseases within flocks.

One such innovative platform is PathoSense's advanced diagnostic system for veterinary infectious diseases, which leverages nanopore sequencing technology (PATHOSENSE, 2024). PathoSense's patented swab design facilitates the collection and preparation of high-quality samples, while the platform’s comprehensive analysis capabilities extend to various animal species and sample types, all while prioritizing sample integrity to ensure reliable laboratory results (PATHOSENSE, 2024). This platform offers several practical advantages for research on chicken gastrointestinal viruses, including simple sampling procedures, rapid result turnaround, and cost-effectiveness, making it an attractive option for poultry health management.

This study aims to investigate the differences in virome genome sequences between young chicks just after arrival and chicks in production three weeks after the first sampling using PathoSense's nanopore sequencing technology. By comparing these sequences, we hope to gain a deeper understanding of how the viral landscape within the chicken gut evolves with age and how this might contribute to the observed age-related susceptibility to specific gastrointestinal diseases. Understanding these age-related differences in virome composition can pave the way for developing targeted diagnostic tools, vaccines, treatments, and biosecurity measures specific to different age groups in poultry management. This, in turn, can lead to improved chicken health, reduced economic losses due to viral diseases, and ultimately, a more sustainable poultry production system.

2. Review of the results of previous research

2.1. The Importance of Flock Health Management and Biosecurity

Chicken meat is one of the most common and popular protein sources worldwide, with its demand steadily increasing across many countries. This growing demand underscores the importance of effective flock health management and biosecurity for profitability. Reports have documented the severe economic impact of poultry diseases, with financial losses for affected hatching egg producers and hatcheries estimated to be as high as $105,000 and $68,000 per 10,000 hens, respectively (LONG et al., 2017). Among the various diseases affecting poultry, enteric diseases are particularly significant, as they often emerge early in a chick's life and can persist within flocks, making them challenging to eliminate and causing long-term damage to productivity.

2.2. Overview of Enteric Diseases in Poultry

Most enteric viral infections occur during the first three weeks of life (SAIF et al., 2020). Enteric diseases are defined as infections affecting the intestinal tract, leading to symptoms such as diarrhea and weight loss, which can result in Malabsorption Syndrome (MSS) or Runting-Stunting Syndrome (RSS) (SAIF et al., 2020; MSD MANUAL, 2024c). Some sources consider MSS, RSS, and White Chick Syndrome (WCS) as part of a broader spectrum of enteric diseases (Table 1) due to their similar clinical symptoms, while others differentiate these conditions based on viral specificity. MSS and RSS are often used interchangeably, with RSS specifically referring to growth retardation, while MSS can arise from both viral and bacterial infections (SAIF et al., 2020; MSD MANUAL, 2024c; DEVENEY et al., 2016). WCS is strictly linked to chicken astrovirus infection and typically affects chicks from the hatchery stage to early life (LONG et al., 2017; SMYTH, 2017).

Table 1. Comparison of Malabsorption Syndrome, Runting-Stunning Syndrome and White Chick Syndrome (Source: MSD MANUAL, 2024c; KUBACKI et al., 2022; SMYTH, 2017)

Syndrome	Virus Involved	Clinical Symptom	Age group
Malabsorption Syndrome (MSS)	Enteroviruses, Parvoviruses, Astroviruses, Caliciviruses, Arenaviruses, Togaviruses, Reoviruses, Rotaviruses	• Uneven growth • Temporary stunting • Permanent runting • Lack of pigmentation in the skin, feet, or beak • Slow feathering • Twisted feathers (“helicopter wings”) • Undigested feed in the feces • Poor feed conversion ratios	Broiler chicks 1–3 weeks old
Runting-Stunning Syndrome (RSS)	Enteroviruses, Parvoviruses, Astroviruses, Caliciviruses, Arenaviruses, Togaviruses, Reoviruses, Rotaviruses	• Uneven growth • Temporary stunting • Permanent runting • Slow feathering	Broiler chicks 1–3 weeks old
White Chick Syndrome (WCS)	Astrovirus Avian Nephritis Virus (ANV) Chichen Astrovirus (CAstV)	• Nephritis, baby chick nephropathy, growth retardation • Growth retardation, kidney disease, White Chicks hatchery disease	Broiler chicks prior or during hatchery

2.3. Transmission and Impact of Enteric Viruses

Chicken astrovirus, the predominant enteric virus in young chicks, significantly influences the gut virome, particularly through vertical transmission from hen to chick (LONG et al., 2017; SMYTH, 2017). Infection can occur as early as the egg or hatchery stage due to vertical transmission, resulting in chicks with compromised immunity, making them less capable of combating existing enteric viruses in their environment (SMYTH, 2017; ADEBIYI et al., 2019). In intensive farming environments, fecal-oral transmission is a major route for viral spread, contributing significantly to endemic infections within flocks. While many enteric viruses are vertically transmitted, horizontal transmission—where chicks infect one another—must not be overlooked, especially in the initial days of life. The combination of poor environmental conditions and the fecal-oral transmission route facilitates swift horizontal transmission, emphasizing the necessity for rapid diagnostics to mitigate financial losses (LIEBHART et al., 2023).

The primary concern regarding viral infections in flocks is not merely the specific virus but also the horizontal spread and viral diversity within the flock, particularly in intensive farming settings. Confinement and suboptimal conditions can exacerbate the situation as chickens mature. Co-infections, where multiple viruses or bacteria compromise the immune system, create a favorable environment for new pathogens to establish. Notably, astrovirus has emerged as a dominant contributor to Runting-Stunting Syndrome (RSS) and is commonly found in younger chickens (SMYTH, 2017).

2.4. Viral Detection in Poultry

Studies have indicated that the presence of certain viruses does not always correlate directly with clinical illness (LIMA et al., 2019). Research has detected similar viral groups in both healthy and sick chickens, suggesting that the mere detection of a virus does not necessarily indicate disease (LIEBHART et al., 2023). This complexity underscores the importance of considering viral strain diversity over sheer quantity in maintaining flock health. Based on the synthesis of current literature and theoretical insights, it appears that viral diversity may play a significant role in the spread of infections within the flock. Recent findings by Grafl et al. (2024) have highlighted correlations between poor weight gain and multiple viral infections in chickens, emphasizing the need for targeted management strategies that prioritize understanding viral diversity to effectively combat enteric diseases in poultry and enhance overall flock health.

Chen et al. (2022) conducted a comprehensive study on enteric viruses in poultry flocks in China, analyzing 145 enteric samples. They identified a total of 191 viruses, with both single (73.1%) and multiple (26.9%) infections detected. The study revealed the presence of various viral pathogens, including Chicken Astrovirus (CAstV) and Avian Nephritis virus (ANV), which were often associated with multiple infections. This work highlights the complexity of enteric viral infections in poultry and underscores the importance of molecular surveillance in understanding the epidemiology of enteric viruses. Such studies provide valuable insights for disease prevention and control and emphasize the need for targeted interventions to improve flock health management.

Astrovirus is a non-enveloped virus with a positive-sense, single-stranded RNA genome of approximately 7.5 kb, characterized by three open reading frames (ORFs) essential for replication and capsid formation (SMYTH, 2017). Chicken Astrovirus (CAstV) is transmitted both horizontally and possibly vertically, with a high contagion rate and a 5-day incubation period (SMYTH, 2017). It primarily targets the enteric tract of young chicks, potentially leading to fatal outcomes due to the lack of specific treatments (SMYTH, 2017). This is particularly concerning given the high prevalence of CAstV in broiler flocks, where it can exacerbate the impact of co-infections with other enteric viruses, such as ANV and rotaviruses. The severity of clinical outcomes is influenced by factors such as viral strain, viral load at the time of infection, and the presence of maternal antibodies. The diversity and evolution of astroviruses are complex (Table 2), but mainly linked to digestive tract illness in various species.

Table 2. Avian Astrovirus Species and Associated Disease (Source: SMYTH, 2017)

Avian Species	Virus	Disease/Condition	Major Tissue Disturbance
Turkey (ICTV designation: Avastrovirus 1)	Turkey astrovirus type 1 (TAstV-1)	Enteritis, growth retardation	Intestine
	Turkey astrovirus type 2 (TAstV-2)	Enteritis, growth retardation, (PEC: poult enteritis complex)	Intestine, bursa of Fabricius, thymus
Chicken (ICTV designation: Avastrovirus 2)	Avian nephritis virus (ANV)	Nephritis, baby chick nephropathy, growth retardation	Intestine, kidney
	Chicken astrovirus (CAstV)	Growth retardation, kidney disease, White Chicks hatchery disease	Intestine, kidney, liver pancreas, spleen
Duck (ICTV designation: Avastrovirus 3)	Duck astrovirus type 1 (DAstV-1)	Hepatitis and variable mortality in young ducks	Liver, kidney, spleen
	Duck astrovirus type 2 (DAstV-2)	Hepatitis and variable mortality in ducklings	Liver, kidney, spleen

Picornaviruses, including Gallivirus and Sicinivirus, are non-enveloped viruses with a single-stranded, positive-sense RNA genome (BOROS et al., 2014). The *Picornaviridae* family consists of 159 species grouped into 68 genera, as of March 2024 (PICORNAVIRIDAE, 2024). The identification of novel strains within this family requires precise analytical methods due to their mutability. The pathogenicity of Picornaviruses, along with co-infective viruses, remains poorly understood due to genetic variation (BULLMAN et al., 2014). However, their rapid evolution and frequent recombination events may explain their presence in seemingly healthy flocks (LAU et al., 2014).

Avian Orthoreovirus (ARV), a member of the *Reoviridae* family, is known as one of the viruses causing RSS. Reoviruses are non-enveloped viruses that contain a segmented (10–12 segments) double-stranded RNA genome (de OLIVEIRA et al., 2021; KUBACKI et al., 2022). Based on identification of ARV in global strains (KOVÁCS et al., 2023), clinical symptoms were segregated into three types: tenosynovitis, RSS, and others. RSS was the second most common symptom observed in the study group. ARV classified into the genus Orthoreovirus, is associated with RSS, MSS, and other gastrointestinal diseases (CHEN et al., 2019). This virus typically affects broilers older than 10 days, likely due to waning maternal immunity (TANG et al., 2015). The clinical signs of ARV can appear as early as 1–2 weeks of age, leading to inflammation of the tibiotarsal-tarsometatarsal joints and rupture of the gastrocnemius tendon, causing varying degrees of lameness in young broilers and breeder chickens (KOVÁCS et al., 2023). Additionally, ARV is frequently found in conjunction with Infectious Bronchitis Virus as a co-infectious agent, followed by Infectious Bursal Disease and adenovirus (KOVÁCS et al., 2023).

Infectious Bronchitis Virus (IBV), belonging to the Gammacoronavirus genus, primarily causes respiratory and urogenital diseases in chickens (AYIM-AKONOR et al., 2018; de SILVA et al., 2021; MSD MANUAL, 2024b). Interestingly, IBV can also lead to uncommon enteric diseases such as RSS. de Silva et al., (2021) successfully isolated the protein responsible for enteric disease from the intestine, named CalEnt, which possesses a unique nucleotide composition. They noted that CalEnt-infected chickens exhibited more severe symptoms than M41-infected chickens with the respiratory tropism.

2.5. Advancements in Diagnostic Methods: Detection Techniques

Several diagnostic methods are available to detect viruses. Molecular detection techniques have revolutionized the identification of smaller viruses, such as parvoviruses, picornaviruses, picornaviruses, and caliciviruses, which are key contributors to RSS (DAY & ZSAK, 2013; LIEBHART et al., 2023). Emerging molecular methods also facilitate the identification of specific strains of astrovirus and rotavirus (LIEBHART et al., 2023). Studies have identified a wide range of viruses, such as *Astroviridae*, *Caliciviridae, Picornaviridae*, *Parvoviridae, and Coronaviridae*, in chickens aged 2–3 weeks (DEVANEY et al., 2016). Another study identified viruses from these same families, along with others like *Reoviridae* and *Smacoviridae,* in both healthy and sick chicks

(KUBACKI et al., 2022). This diversity highlights that the virome of chickens is dynamic, shaped by various environmental, biological, and management factors.

Understanding the historical challenges in detecting viruses is also crucial. Traditionally, virus detection in the gut posed significant challenges, relying heavily on techniques such as electron microscopy, cell culture, and immunohistology (SAIF et al., 2020; GUY, 1998). Although these methods remain in use today, they have limitations in identifying specific viral strains (LIEBHART et al., 2023). The advent of genomic sequencing has revolutionized the field by enabling researchers to uncover phylogenetic differences among viruses and within virus families (LIMA et al., 2017; CHEN et al., 2022). For instance, studies have indicated that chicken astrovirus and avian nephritis virus stemmed from turkey astrovirus, as identified through genetic sequencing (SMYTH, 2017).

Metagenomic analysis has further advanced our understanding of enteric viruses, revealing complex viromes and previously unrecognized viral families in chickens (CHEN et al., 2022; KUBACKI et al., 2022; LIMA et al., 2019). This research underscores the significance of understanding the diversity of specific viral strains within flocks, which is crucial for effectively managing endemic infections and informing the development of vaccines and other treatment strategies (PETRONE-GARCIA et. al, 2021). It also underscores the need to continually revise virus taxonomies as new strains are discovered (GORAICHUK et al., 2021). Novel virus species are investigated to understand the diversity of virus species, mutation, recombination, and their contributions to the molecular epidemiology of viruses (GORAICHUK et al., 2021). Despite advancements in diagnostic methods to identify various viral strains and genomic sequences, these techniques require specific laboratory conditions and highly skilled technicians, making them impractical for routine flock management (LIEBHART et al., 2023).

2.6. Biosecurity Measures and Management Strategies

In response to the ongoing threat of enteric viruses, farmers have implemented measures to enhance biosecurity, including the use of vaccines and nutritional supplements to prevent disease outbreaks (LIEBHART et al., 2023). While education on biosecurity measures is crucial, researchers are increasingly focused on refining diagnostic techniques to swiftly identify viral infections and develop targeted treatments. Improved hygiene protocols, such as the use of protective clothing and regular pest control, can also help reduce the overall viral burden within flocks and spread between the flocks (GRAFL et al., 2024). Therefore, biosecurity measures must not be overlooked when addressing enteric diseases in chickens.

3. Material and Methods

3.1. Monitored animals

Monitoring was done on two broiler farms with Ross 308 genetics delivered from the same hatchery, at 5 and 25 days of age. Fresh morning culls were delivered to the Department's clinic and samples were taken immediately after delivery.

3.2. Sampling

After necropsy and examination of the carcasses, intestinal samples, from duodenum to ileum, were randomly swabbed using specifically designed swab with a tube in a PathoSense kit (PathoSense, Ghent, Belgium) (Figure 1). Immediately after sampling swab was washed and mixed with preserving medium and such prepared samples were stored on 4°C before sending. Each kit was used for sampling of 5 animals per farm, so in total 4 merged samples were taken and sent for sequencing.

3.3. Sequencing

Each sample is marked with a specific QR code which was uploaded into the mobile PathoSense platform (PathoSense, Ghent, Belgium) with the short anamnesis and description of sampled material. Sequencing was performed using nanopore methodology (Oxford Nanopore Technologies, Oxford, UK). After sequencing, qualitative and quantitative results were uploaded into personal mobile platform (Figure 1).

Figure 1. PathoSense sampling kit (swab, tube and syringe with preserving medium) marked with unique QR code and following results on the mobile platform.
(Source: https://www.pathosense.com/en)

4. Results

The results of this study are summarized in Table 3 and Figures 2 to 4. From the gained results, it was observed that at 5 days of age, only astrovirus was detected on both farms (Table 3, Figure 2). By 25 days of age, there was an increase in viral diversity, with additional viruses detected in both farms. In Farm 1, astrovirus, Avian Orthoreovirus (ARV), and Infectious Bronchitis Virus (IBV) were identified at 25 days of age (Figure 3). Similarly, in Farm 2, four additional viruses were detected alongside astrovirus: Chicken Calicivirus, Gallivirus, IBV, and Sicinivirus (Figure 4).

Table 3. Intestinal virome found in sampled chickens at 5 and 25 days of age on Farm 1 and 2

Farm 1		Farm 2	
Day 5	Day 25	Day 5	Day 25
Astrovirus	Astrovirus	Astrovirus	Astrovirus
	Avian orthoreovirus		Chicken calicivirus
			Gallivirus
	Infectious bronchitis virus		Infectious bronchitis virus
			Sicinivirus

Beside the qualitative results showing the viral genus, quantitative results also report the viral concentrations, categorized into low, medium, and high, with each category represented numerically by units of 5 for ease of interpretation. Notably, the loads of astrovirus increased in Farm 1 from day 5 to day 25 (Figure 2 and 3), while it decreased in Farm 2 over the same period (Figure 4). IBV was found in both farms at 25 days old, with a lower loads in Farm 1 compared to Farm 2 (Figure 3 and 4). Chicken Calicivirus, Gallivirus, and Sicinivirus were detected at medium loads in Farm 2 at 25 days old (Figure 4), although these viruses were absent at 5 days of age. Importantly, all viruses detected were RNA viruses (Table 4).

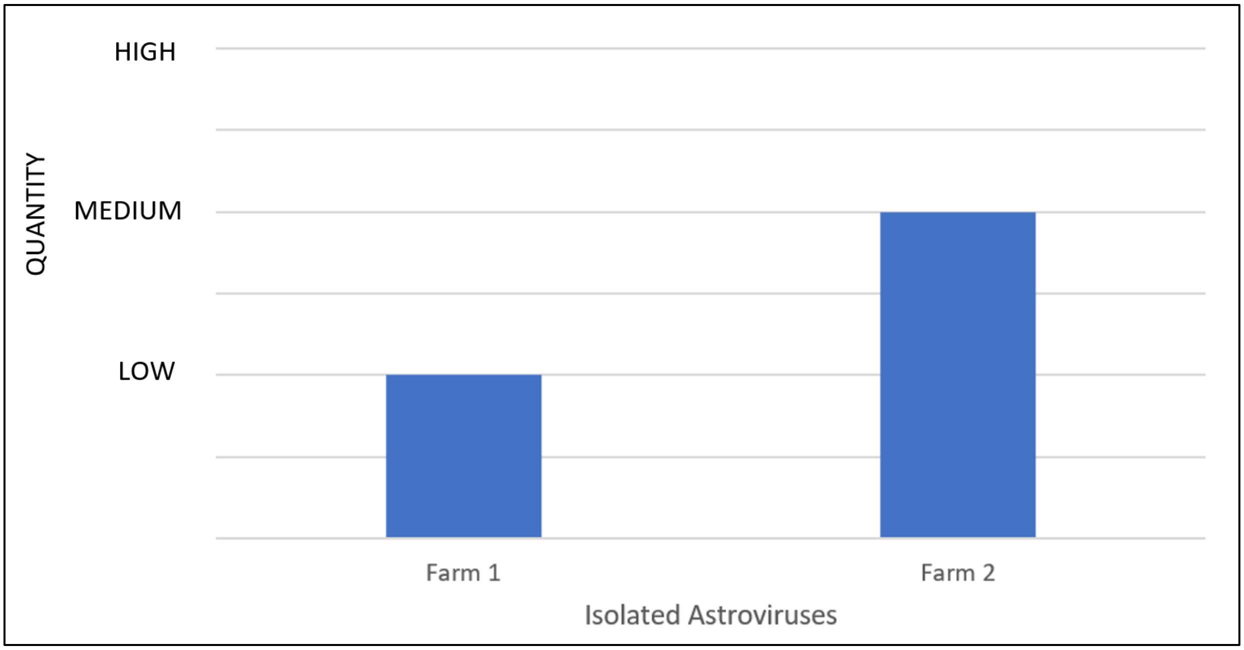

Figure 2. Quantitative virome results of first sampling on Farm 1 and 2 at 5 days of age

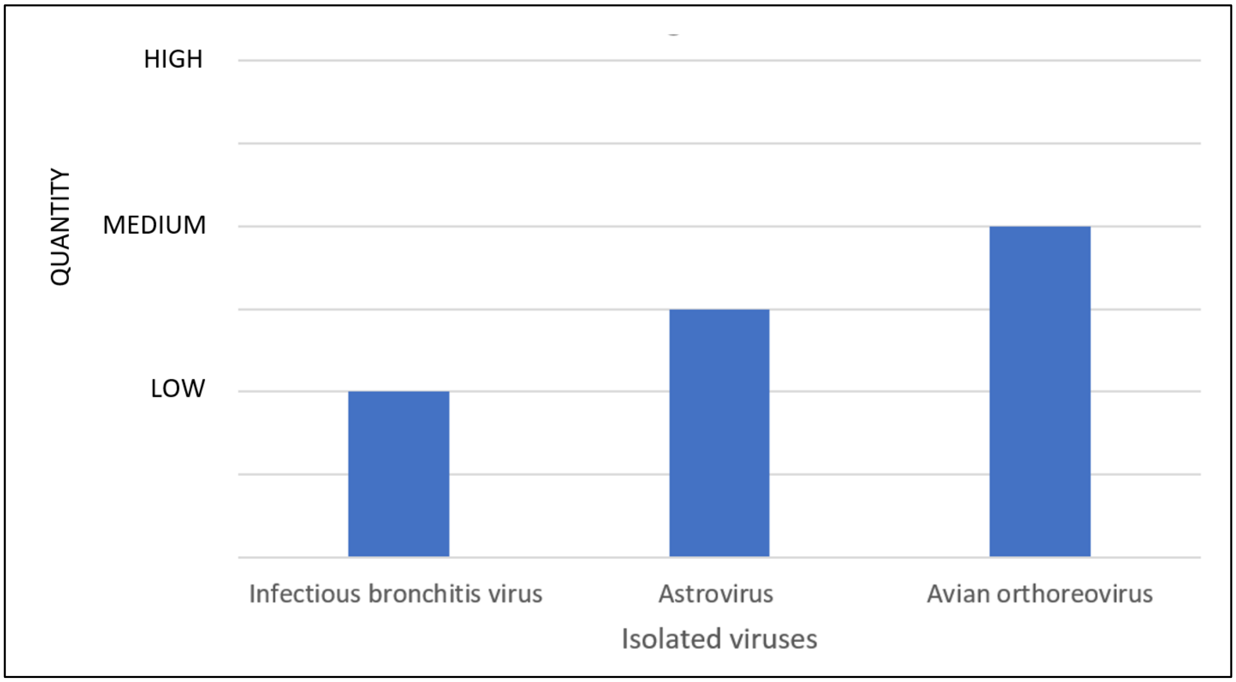

Figure 3. Quantitative virome results of sampling at 25 days of age on Farm 1

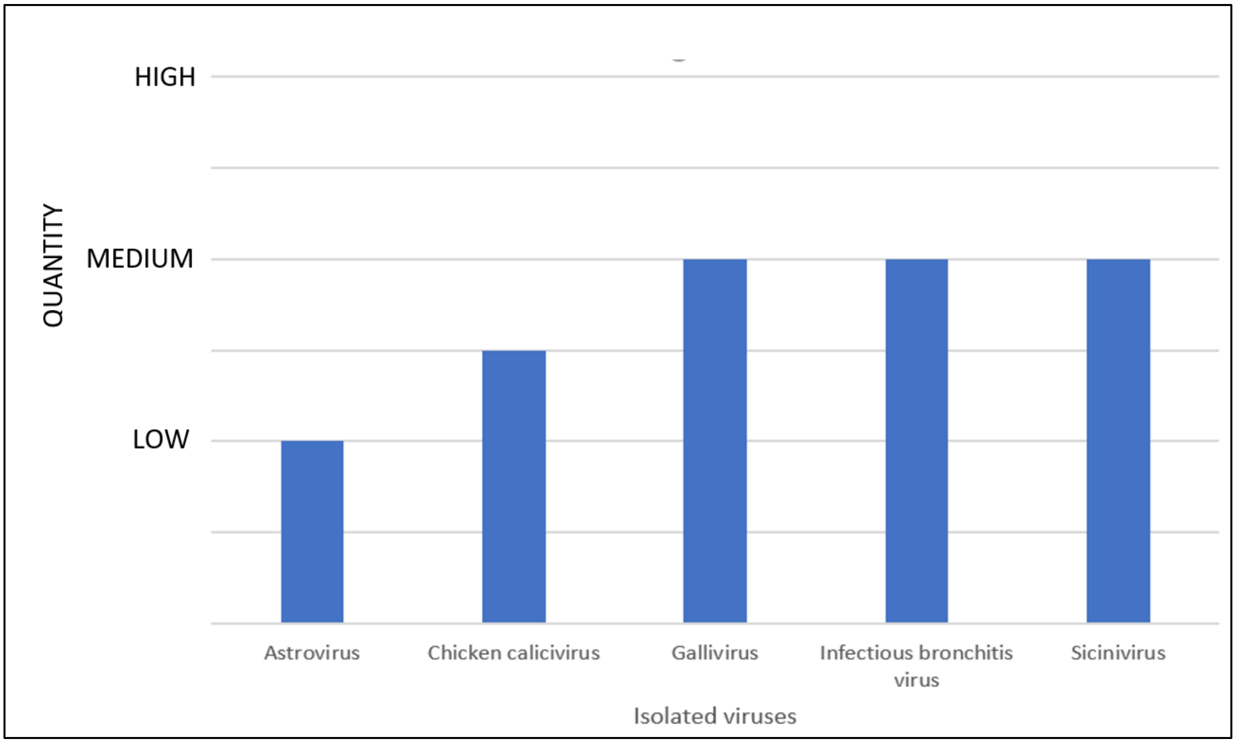

Figure 4. Quantitative virome results of sampling at 25 days of age on Farm 2

Table 4. Detected virus families

Genome	Family	Genus
RNA	*Astroviridae*	Astrovirus
	Reoviridae	Avian Orthoreovirus
	Coronaviridae	Infectious bronchitis virus
	Caliciviridae	Chicken Calicivirus
	Picornaviridae	Gallivirus
	Picornaviridae	Sicinivirus

5. Discussion

Broilers are particularly susceptible to enteric viruses, leading to Malabsorption Syndrome (MSS) or Runting-Stunting Syndrome (RSS), especially under suboptimal hygiene and management conditions (de OLIVEIRA, 2021; KUBACKI, 2022). These diseases result in significant economic losses due to poor feed conversion, uneven growth, and related symptoms (MSD MANUAL, 2024c). The primary viral families associated with MSS include *Adenoviridae*, *Caliciviridae, Circoviridae, Parvoviridae*, *Picornaviridae*, *Reoviridae, Astroviridae*, and others (DEVANEY et al., 2016; LIMA et al., 2017).

Metagenomic studies have become instrumental in characterizing the enteric virome in poultry, providing insights into the diversity and dynamics of viral populations. Recent research utilizing high-throughput sequencing has identified numerous viral strains that conventional diagnostic methods may overlook (DEVANEY et al., 2016; LIMA et al., 2017). This technique allows for a more comprehensive understanding of viral interactions and their potential impact on avian health. For instance, studies employing metagenomics have documented the co-occurrence of multiple viral species in broiler flocks, which may contribute to disease syndromes such as MSS and RSS (DEVANEY et al., 2016). Such findings underscore the importance of utilizing advanced genomic techniques to inform effective disease management strategies. While the etiological role of some viruses remains unclear, as they are identified in both sick and healthy chicken guts, it is noteworthy that viruses found in metagenomic analysis in the gut can also be present in normal gut microbiota (LIMA et al., 2019).

Previous studies have identified both DNA and RNA viruses in broilers as young as 10 days old; however, this study detected only RNA viruses in both 5- and 25-day-old groups (DEVANEY et al., 2016; GRAFL et al., 2024). Other studies have identified DNA viruses such as parvovirus and adenoviruses, known for their association with enteric diseases. The identification of astrovirus at a young age in this study is consistent with the literature, which generally finds both Chicken Astrovirus (CAstV) and Avian Nephritis Virus (ANV) together (SMYTH, 2017; ADEBIYI et al., 2019). In this study, only CAstV was found at 5 days of age, while other research has reported multiple viruses present at this stage (DEVANEY et al., 2016). CAstV, a member of the *Astroviridae* family, is highly prevalent in young broiler flocks or hatcheries (KAITHAL et al., 2016; SMYTH, 2017). This finding is not surprising, as CAstV is a frequently detected virus strain with a higher prevalence than ANV; its resilience may contribute to its persistence in later life stages (KAITHAL et al., 2016; ADEBIYI et al., 2019; CHEN et al., 2022).

Although this study did not identify specific strains or viral doses, the higher quantitative units detected in Farm 2 suggest a greater viral burden as the chicks age, possibly indicating a compromised immune system. This finding is critical, as it highlights the need for effective management strategies to monitor and control CAstV infections, especially in farms with higher viral loads. The alignment of increased viral load with the observation of widespread CAstV infection in the flock further underscores the potential for CAstV to weaken the chicks' immunity and intestinal integrity, facilitating subsequent infections with other related viruses.

The increased viral load at 25 days of age in both farms, particularly in Farm 2, where higher quantitative units of Chicken Calicivirus, Sicinivirus, and Infectious Bronchitis Virus (IBV) were identified, underscores the possibility of ongoing viral infections and their mutual pathogenicity (KUBACKI et al., 2022). Therefore, understanding the dynamics of CAstV within the broader virome is essential for developing targeted interventions to enhance flock health and mitigate the risks associated with viral diseases.

Notably, Picornaviruses, including Gallivirus and Sicinivirus, and Caliciviruses were found to be associated with RSS later than other virus groups, such as astrovirus and rotavirus (KUBACKI et al., 2022). The study by de Oliveira et al. (2021) identified Picornaviruses in histopathology from clinically symptomatic broiler chickens, indicating their environmental stability and ability to survive in various hygiene conditions. This was also supported by Kubacki et al. (2022), who noted that the detection of these viruses does not necessarily indicate poor farm hygiene, as they can be found in both healthy and diseased chickens. The random sampling method employed in this study does not conclusively determine the hygienic conditions of Farm 2; however, the presence of CAstV at 5 days of age likely compromised the gut lining and immune system of the broilers. Additionally, the subsequent infections of calicivirus and picornaviruses confirm that Farm 2 can be classified as possibly experiencing malabsorption syndrome (MSS), characterized by diverse enteric viral infections affecting the flock. Picornaviruses, including Gallivirus and Sicinivirus, were found in conjunction with CAstV, indicating their role in complex viral interactions within the flock. The ability of these viruses to persist in both healthy and diseased populations poses challenges for disease management. The presence of diverse viral strains underscores the necessity for vigilant biosecurity measures and continuous monitoring of flock health.

Interestingly, Avian Orthoreovirus (ARV) was detected exclusively in 25-day-old chicks in Farm 1, exhibiting the highest quantitative units among the viruses identified at this age, although this is still considered an intermediate level of infection. Probably due to waning maternal immunity (TANG et al., 2015), this virus starts to affect broilers older than 10 days, which possibly explains its absence in the 5-day-old chicks. The detection of ARV at 25 days of age, combined with its absence at 5 days in Farm 1, suggests that while maternal immunity initially provided protection, potential hygiene deficiencies may have facilitated the virus's proliferation later. This finding aligns with Kubacki et al. (2022), who emphasized the association of ARV with poor hygiene environments. Moreover, the presence of ARV alongside other identified pathogens underscores the importance of addressing hygiene and management practices to mitigate the impact of multiple viral infections in broiler flocks. Continued sampling and environmental improvements are recommended to address these concerns.

A critical observation from the research is that current commercial vaccine strains for ARV do not align with the emerging ARV strains present in the flock. Chen et al., (2019) noted that the current commercial vaccine strain, LY383, is vertically transmitted to the progeny but may not effectively protect against newly emerging strains. This mismatch underscores the need for continuous monitoring of circulating ARV strains and the adaptation of vaccination strategies to ensure effective protection of broiler flocks.

Infectious Bronchitis Virus (IBV) was detected in both farms at 25 days of age, highlighting its prevalence across the studied populations. The detection of IBV at this age may be attributed to the presence of maternal antibodies at 5 days, which may have temporarily protected the chicks, but also with vaccination against IB. However, as these antibodies wane, the flock becomes more susceptible to IBV infection. The emergence of IBV at this later age, particularly in Farm 2, where viral loads were higher, could be indicative of an outbreak stemming from stressors such as poor management or environmental factors. On the other hand, it could be result of progressive replication of vaccine strains as well.

The prevalence of IBV in older broilers could indicate its role in enteric disease syndromes like RSS. The detection of IBV correlates with factors such as maternal antibody decay and environmental stressors, emphasizing the importance of addressing management practices to mitigate the risk of viral outbreaks. As vaccinated against IBV, this should be the right reason for the presence of IBV in the sample, but typing of detected strains could elucidate their source and possible pathogenicity.

Overall, while only one virus was detected at 5 days of age, three viruses were found in Farm 1 and five in Farm 2 at 25 days, suggesting a greater susceptibility to viral infection in the latter. Although a direct comparison of management practices between the farms is not necessary for this study, these findings indicate that Farm 2 had a higher viral load at this age. As noted by Grafl et al. (2024), the poor health condition of chickens is associated with an increase in the number of viruses present. Therefore, it is likely that the viral load is increased in Farm 2, considering their lower immune condition. This observation underscores the importance of continued monitoring of viral infections and the potential need for enhanced biosecurity measures to improve flock health.

The results confirm the hypothesis of possible vertical transmission and early infection of chicks with intestinal viral pathogens, leading to later viral diversification. This underscores the importance of detailed monitoring of parent flocks and their offspring to prevent vertical transmission and improve vaccination programs, ultimately enhancing chicken protection, health, and overall biosecurity in integrated systems.

Epidemiological studies play a critical role in understanding the transmission dynamics of enteric viruses in poultry populations. For instance, studies conducted in various geographic regions have documented the prevalence of enteric viruses and their association with specific management practices (VAN LIMBERGEN et al., 2018; GRAFL et al., 2024). These investigations reveal how environmental factors, biosecurity measures, and flock management contribute to the incidence and spread of viral infections. Additionally, understanding the epidemiology of enteric viruses aids in the development of targeted vaccination and intervention strategies, as evidenced by outbreaks linked to specific viral strains (da SILVA et al., 2021; GORAICHUK et al., 2021; CHEN et al., 2019). This emphasizes the necessity for continued surveillance and data collection to mitigate the impact of viral pathogens in poultry farming.

The study underscores the critical role of epidemiological insights in understanding viral transmission dynamics within poultry. The findings advocate for enhanced biosecurity measures and targeted vaccination strategies to improve flock health and reduce economic losses associated with enteric viral infections. Continued surveillance is essential to adapt to emerging viral strains and maintain effective disease management.

PathoSense is a valuable tool utilized in this study to assess viral load through swab samples, categorizing the results into low, medium, and high viral loads. This method offers several advantages over traditional PCR techniques, including cost-effectiveness, speed, and ease of sampling, as it does not require highly trained staff for operation. However, one significant limitation is that PathoSense does not easily identify specific viral strains that may be circulating within a flock. While PathoSense efficiently provides a quantitative assessment of viral presence, it falls short in detecting novel strains that could lead to severe outbreaks on farms. In contrast, specialized strain research is crucial for accurately identifying circulating viral strains and developing targeted vaccines, but this approach is typically costlier and requires skilled personnel.

Despite its limitations, PathoSense remains a practical solution for routine flock management. The ability to identify the number of viruses present and their quantitative loads can inform management strategies effectively. Thus, for farms prioritizing operational efficiency and cost-effectiveness, PathoSense serves as a complementary tool, enabling the monitoring of viral loads, wild and vaccinal, without the extensive resources required for strain identification.

6. Conclusion

In conclusion, this study utilized the PathoSense method, a quantitative tool for detecting virome in the gut, to compare the enteric virome in broiler chickens at 5 and 25 days of age across two farms. Initially, only one virus was detected in both farms at 5 days of age, but by 25 days, a greater variety of viruses—three in Farm 1 and five in Farm 2—were identified. While the PathoSense method has limitations, including its inability to gain good quality sequences at lower doses and differentiate between viral strains, the findings still provide significant evidence of both vertical transmission and viral diversification at later ages. These results highlight the critical need for stringent biosecurity and improved management practices in broiler production to prevent the spread of viral infections. It is a simple and rapid method for basic chickens' microbial monitoring on a farm, wild and vaccinal, and an excellent bioindicator for management and biosecurity practices and post-application monitoring for various treatments.

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8. Sažetak

Virom tovnih pilića u konvencionalnoj proizvodnji

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Tovni pilići igraju važnu ulogu u globalnoj sigurnosti hrane, posebice u proizvodnji mesa. Složeni gastrointestinalni mikrobiom kod pilića ključan je za cjelokupno zdravlje, djelujući na metabolizam i imunitet, no utjecaj virusnih infekcija ostaje nedovoljno istražen. Ovaj rad analizira virom tovnih pilića dobi 5 i 25 dana na dvije farme, koristeći PathoSense dijagnostički kit i platformu za dokaz i kvantifikaciju virusa sekvenciranjem nanopore postupkom. U dobi od 5 dana identificiran je samo Astrovirus na obje farme. Nasuprot tome, nakon 25 dana, na Farmi 1 identificirano je tri skupine virusa, Astrovirus, ptičji Orthoreovirus i virus zaraznog bronhitisa, dok je na Farmi 2 utvrđeno pet skupina virusa, Astrovirus, kokošji Calicivirus, Gallivirus, Sicinivirus i virus zaraznog bronhitisa. Ovi nalazi upućuju na ranu i intenzivnu infekciju vertikalnim prijenosom, uz kasnije horizontalno širenje i diverzifikaciju virusa. Slabljenje imuniteta u ranoj dobi, djelovanjem niza čimbenika, moglo bi olakšati širenje i diverzifikaciju virusa. Ovo istraživanje naglašava nužnost pojačanih biosigurnosnih mjera i ciljanih intervencija u kontroli zdravlja peradi, od roditeljskih jata, valionica do proizvodnih farmi, a korištena jednostavna i brza metoda mogla bi biti dobar bioindikator za poboljšanje zdravlja jata i ublažavanje ekonomskih gubitaka djelovanjem virusnih bolesti.

Ključne riječi: tovni pilići, crijevni virom, nanopore sekvenciranje, PathoSense

9. Summary

Virome of broiler chickens in conventional production

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Broiler chickens play a vital role in global food security, particularly in meat production. The complex gastrointestinal microbiome in chickens is crucial for overall health, affecting metabolism and immunity, however, the impact of viral infections remains insufficiently studied. This study investigates the virome of 5 and 25 days old broilers from two farms, utilizing the PathoSense diagnostic tool and platform to identify and quantify viral loads using nanopore sequencing methodology. At 5 days of age, only Astrovirus was identified in both farms. In contrast, at 25 days, on Farm 1 three viruses were identified, Astrovirus, avian Orthoreovirus and infectious bronchitis virus, while on Farm 2 five viruses were revealed, Astrovirus, chicken Calicivirus, Gallivirus, Sicinivirus and infectious bronchitis virus. These findings suggest an early and intensive infection via vertical transmission and later horizontal spread and viral diversification. The compromise of the immunity at an early age could facilitate viral spread and diversification. This study highlights the necessity for enhanced biosecurity measures and targeted interventions in poultry management, from parent flocks, hatcheries to production farms, and the used simple and rapid method could be a good bioindicator for improvement of flock health and mitigation of the economic impacts of viral diseases.

Key words: broiler chickens, intestinal virome, nanopore sequencing, PathoSense

10. Curriculum Vitae

Eunhee Lee has degree in environmental engineering and has worked as wastewater treatment and soil remediation projects. She is currently a 6th-year student in the Faculty of Veterinary Medicine at the University of Zagreb. Her passion for animals has shaped her career path, but beyond this, she enjoys watching crime documentaries, crocheting, South Park, and reading sci-fi. Now, she is especially into the *Three-Body Problem* trilogy. Over the past six years, she has watched the entire South Park series three times and crocheted an array of items, including a muffler, summer and winter hats, Christmas ornaments, and other basics—just in case of a doomsday emergency. Recently, she has been fascinated by the concept of hibernation technology from the Three-Body Problem, dreaming of waking up 100 years in the future to discover whether the infectious diseases she's studied have become obsolete or if genomic cure medications are the norm. She imagines herself lecturing a new generation on the hard-earned knowledge of fundamental veterinary medicine.

Ms. Lee has also developed a deep interest in poultry health management, particularly focusing on enteric viral infections in chickens. Her ultimate goal is to manage her own poultry farm, where she hopes to apply her knowledge to improve animal health and production practices.