UNIVERSITI PUTRA MALAYSIA

SEROPREVALENCE OF CHICKEN ASTROVIRUS IN COMMERCIAL BROILER BREEDERS IN PENINSULAR MALAYSIA

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SEROPREVALENCE OF CHICKEN ASTROVIRUS IN COMMERCIAL BROILER BREEDERS IN PENINSULAR MALAYSIA

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CERTIFICATION

It is hereby certified that I have read this project entitled "Seroprevalence of Chicken Astrovirus in Commercial Broiler Breeders in Peninsular Malaysia", by Mohamad Syazwan Syafiq bin Mohd Bokhari and in my opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999- Final Year Project.

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DEDICATIONS

This project paper is dedicated to

whom it may concern.

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In the name of Allah, the most Merciful and the most Compassionate. All praises upon Him for giving me the patience, courage and strength in my journey towards completing this project.

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Abstrak daripada kertas projek yang dikemukakan kepada Veterinar Fakulti Perubatan untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Tahun Akhir

SEROPREVALENS ASTROVIRUS AYAM DALAM AYAM PEDAGING PEMBIAK BAKA DI SEMENANJUNG MALAYSIA

Oleh

MOHAMAD SYAZWAN SYAFIQ BIN MOHD BOKHARI

2020

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Industri ayam merupakan sektor haiwan ternakan terpenting di Malaysia, yang menghasilkan sumber protein yang terjamin dan kos efektif dalam menampung kepesatan peningkatan populasi di dalam negara. Selain daripada peningkatan kos produksi lantaran kenaikan harga makanan ayam, penyakit berjangkit, terutamanya kemunculan dan kemunculan semula wabak penyakit juga menjadi ancaman nyata terhadap industri ayam komersial. Virus astro ayam (CAstV) merupakan salah satu daripada kemunculan baru virus yang menjangkiti unggas di peringkat global. Kes jangkitan penyakit ini dalam kalangan ladang ayam komersial telah dilaporkan di merata dunia, termasuklah negara pengeluar ayam utama dunia seperti Amerika Syarikat, China, Brazil, India dan juga Eropah. Walau bagaimanapun, prevalens CAstV dalam kalangan ayam komersial di serata Asia Tenggara termasuklah Malaysia masih belum dikaji dengan baik. Kajian ini bertujuan untuk menentukan prevalens sera dalam kalangan ladang pembiak baka ayam komersial di Semenanjung Malaysia. Sejumlah 415 sera ayam pedaging pembiak baka yang berlainan umur yang diarkibkan di Unit Diagnostik Avian, Institut Biosains, Universiti Putra Malaysia telah digunakan di dalam kajian ini. Ujian enzim-pengerap immuno (ELISA) tidak langsung yang mengesan CAstV kumpulan B komersial telah digunakan untuk menentukan tahap antibodi terhadap CAstV. Titer antibodi daripada ELISA kemudiannya dianalisis menggunakan Ujian ANOVA sehala. Kajian menunjukkan bahawa kesemua sampel didapati positif terhadap CAstV. Secara statistik, terdapat perbezaan nyata ($p \leq 0.05$) terhadap titer antibodi dalam kalangan kumpulan ayam berumur 40 hingga 50 minggu jika dibandingkan kepada kumpulan ayam berumur kurang daripada 40 minggu dan lebih daripada 50 minggu. Walau bagaimanapun, titer antibodi ayam dalam lingkungan umur kurang daripada 40 minggu dan lebih daripada 50 minggu tidak berbeza secara signifikan. Lantaran ketiadaan vaksinasi terhadap CAstV di Malaysia, sejajar dengan kesemua ladang tidak mempraktikkan vaksinasi terhadap CAstV, membuktikan bahawa insiden jangkitan CAstV yang tinggi adalah kerana pendedahan kepada virus ini. Tuntasnya, kajian ini telah memberikan sedikit gambaran dan maklumat yang berharga untuk digunakan oleh kajian lanjutan dalam membangunkan langkah-langkah pengawalan dan pencegahan terhadap CAstV di ladang ayam di Malaysia.

KATA KUNCI: Virus astro ayam, prevalens sera, Ujian Enzim-Pengerap Immuno, ayam pedaging pembiak baka

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

SEROPREVALENCE OF CHICKEN ASTROVIRUS IN COMMERCIAL BROILER BREEDERS IN PENINSULAR MALAYSIA

By

MOHAMAD SYAZWAN SYAFIQ BIN MOHD BOKHARI

2020

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The poultry industry is the most important livestock sector in Malaysia, producing a reliable and cost-effective animal protein source supporting the country's growing population. Besides the rising production cost due to the increase in poultry feed price, infectious diseases, especially emergence and re-emergence diseases, also pose significant threats to the commercial poultry industry. Chicken astrovirus (CAstV) is one of the new emerging viruses affecting poultry birds globally. This disease has been reported in commercial poultry farms worldwide, including in several major poultry producing countries such as the United States of America, China, Brazil, India, and Europe. However, the prevalence of CAstV in commercial chickens in South-East Asia countries, including Malaysia, is not well studied. This study aims to determine the seroprevalence of CAstV in selected commercial broiler breeder farms in Peninsular Malaysia. A total of 415 archived serum samples from broiler breeders of different ages available at the Avian Diagnostic Unit, Institute of Bioscience, Universiti Putra Malaysia, were used in this study. A commercially available indirect enzyme-linked immunosorbent assay (ELISA) that detects Group B CAstV was used to determine the antibody levels against CAstV. The ELISA antibody titers were then analysed by using One-way ANOVA. The study revealed all the tested samples were tested positive for CAstV. Statistically, there was a significant difference ($p \leq 0.05$) in the antibody titers of chickens of age group 40 to 50 weeks compared to the chicken of age group less than 40 weeks age and chicken of age group more than 50 weeks of age. However, the antibody titer of chicken of the age group less than 40 weeks and more than 50 weeks were not significantly different. Since the vaccination against CastV is not available in Malaysia, and all the farms do not practice vaccination against CastV, the high incidence of CAstV indicates the flocks have been exposed to the virus. Thus, this study has provided some insight and valuable information for further research towards developing proper prevention and control measures of CAstV in poultry farms in Malaysia.

KEYWORDS: Chicken Astrovirus, Seroprevalence, Enzyme-linked immunosorbent assay, Broiler breeders

1.0 INTRODUCTION

Chicken astroviruses (CAstV) are small round, non-enveloped, single-stranded RNA viruses typically < 35 nm in diameter with a star-like morphology (Reynolds and Schultz-Cherry, 2008). The virus infects a variety of poultry species, especially chicken, turkey and ducks (Ali *et al.*, 2020). In addition, Smyth (2017) stated that CAstV infections are commonly found in broiler chickens and are highly associated with the diseases of young birds and hatchery disease. Other than that, the primary tissue tropism of the virus is in the intestine, kidney, liver, pancreas and spleen, causing growth retardation, kidney disease and White Chicks hatchery disease. Besides, CAstV is one of the causative agents often associated with enteritis problems in chickens (Mettifogo *et al.*, 2013).

The incidence of CAstV infection is commonly due to horizontal transmission via fecal-oral route (Xue *et al.*, 2017). However, the virus can also be transmitted vertically from infected in-lay parents since there was evidence of seropositive cases in 1-day-old chicks and even dead-in-shell chicks (Todd *et al.*, 2009).

Several diagnostic techniques are available for confirmatory diagnosis, including virus isolation, serologic methods, electron microscopy, RT-PCR, and real-time RT-PCR (Long *et al.*, 2018). Although virus isolation and electron microscopy can be used as diagnostic tools, the virus is difficult to culture, and it may give inconclusive results since all astrovirus and some other viruses may resemble each other (Canelli *et al.*, 2012). Therefore, convenient serological tests such as virus neutralization and enzyme-linked immunoadsorbent assay (ELISA) can be used other than RT-PCR and real-time RT-PCR.

A study conducted showed that the economic impacts of astrovirus infections in the poultry industry are difficult to be assessed (Reynolds and Schultz-Cherry, 2008). There is limited information on the economic losses caused by CAstV to the poultry industry; its implication on financial losses arises from growth depression, including uneven growth and runting-stunting syndrome due to increased culling, low feed conversion ratio and lower uniformity at slaughter (Oluwayelu and Todd, 2012). As of present, no significant countermeasure on these issues as there is no vaccine currently available against CAstV infection.

Currently, hygiene and biosecurity are the only ways to minimise CAstV infection (Smyth, 2017). Recently, sequence analysis confirmed the presence of group B CAstV in poor performance broiler farms in Malaysia (Raji *et al*., 2019). A pathogenicity study on some of the isolated CAstV in specific-pathogen-free (SPF) chickens confirmed the virus is pathogenic and can cause clinical signs and lesions associated with enteric infection (Raji, 2021). However, the seroprevalence of the CAstV in commercial chickens in Malaysia has not been studied.

1.1 Hypothesis

The hypothesis of this study is the seroprevalence of chicken astrovirus (CAstV) in commercial broiler breeders in Peninsular Malaysia is high.

1.2 Objectives

This study is aimed to determine the seroprevalence of group B CAstV in broiler breeders of different ages in selected commercial poultry farms in Peninsular Malaysia. Moreover, this study is the first serological prevalence study of CAstV in Malaysia. Undoubtedly, the data obtained in this study will help to determine the current status of CAstV and precursor for further research.

2.0 LITERATURE REVIEW

2.1 Chicken Astrovirus

Chicken astrovirus (CAstV) is a small, rounded, non-enveloped virus that typically has 28-30 nm in diameter and a positive sense, single-stranded RNA genome with a length of 7.5 kb (Ali *et al*., 2020). The name astrovirus derives from a Greek word "astron", means star, referring to its 5-6 pointed star-like surface projections under negatively stained electron microscopy (Sharma *et al*., 2017). The virus is a member of *Avastrovirus* or commonly known as avian astrovirus and it belonged to the family of *Astroviridae* (Matsui & Greenberg, 2001). Previously, CAstV was described as "enterovirus-like virus" (ELV) due to its common characteristics to the virus from the genus Enterovirus and family Picornaviridae (Smyth, 2017).

Genome sequencing of the CAstV revealed that it shared similar genetic organization with other astroviruses where it is composed of merely three open reading frames (ORF) where the first two, ORF 1a and ORF 1b encoding for non-structural protein including proteases and an RNA dependent RNA polymerase, respectively. On top of that, the third ORF, ORF 2 encodes for the capsid protein; the highest variable part of the genome, especially at the 3' half of the ORF that encoding for the capsid's outer surface (star-like capsid spikes), playing a role in the interaction between the virus and host immune system (Smyth, 2017).

Studies have indicated two different serogroups of CAstV (McNulty *et al*., 1990; McNeilly *et al*., 1994) that showed some degree of cross-reactivity with heterologous antisera (Smyth, 2017). The finding was further supported with the genotyping study based ORF 2 gene by clustering the virus into two CAstV groups, group A and group B. Within these two groups, CAstV group A is comprised of three different subgroups; Ai, Aii and Aiii, while group B is divided into two subgroups; Bi and Bii (Smyth *et al*., 2012). Furthermore, phylogenetic analysis based on the ORF2 gene able to classify the viruses based on the particular diseases they cause.

Currently, sequence analysis confirmed the presence of CAstV in poor performance broiler farms in Malaysia (Raji et al., 2019). A pathogenicity study on some of the isolated CAstV in specific-pathogen-free (SPF) chickens confirmed the isolated virus is pathogenic and can cause clinical signs and lesions associated with enteric infection (Raji, 2021).

2.2 Poultry Production in Malaysia

The poultry industry is one of the most important livestock sectors in Malaysia. According to the Department of Statistics Malaysia, in 2017, the poultry consumption per capita was about 52 kilograms, which constitutes 98.2% self-sufficiency ratio (SSR). According to Jamaludin (2013), the high demand for poultry as a protein source is because there is no religious restriction in the consumption of poultry meat compared to beef and pork and to a lesser extend mutton, which may not be suitable to specific segments of the population.

In 2017, the Department of Veterinary Services, Malaysia recorded there are approximately 163,631,796 broiler populations in Peninsula Malaysia, with Johor as the largest producing state followed by Kedah and Perak. The broiler industry structure comprised breeding farm; Grand Parent Stock (GPS) and Parent Stock (PS), hatcheries including broiler farms meant to produce chickens for meat. The GPS is entirely imported and bred to be PS, which is utilized in producing eggs at broiler breeder farms meant to produce day-old chicks (DOC) upon hatching. Usually, hatcheries are owned by the broiler breeder farm itself. The DOC is then supplied to broiler farms to produce table chickens (Jamaludin, 2013).

The broiler industry in Malaysia can be divided into two categories, conventional farm and commercial farm. The conventional farm is a farm that belongs to independent entrepreneurs, while the commercial farm is a business with cooperation among integrator and farmer based on contract farming, and it is the most common practice in Malaysia (Bahri *et al*., 2019).

Over the past few decades, infectious disease, including viral infection, has become one of the causes of economic losses in this industry. On top of that, the identification process of the causative agent and detection of specific antibody responses based on clinical manifestation are challenging caused by concurrent infection and inappropriate use of vaccines. Presently, the poultry business is compromised by more virulent endemic virus infection or by an exotic and emerging disease that can lead to a significant economic loss (Omar, 2013)

2.3 Chicken Astrovirus Infection

CAstV infections are common in broiler chickens and are highly associated with the diseases of young birds and hatchery disease. Typically, the infections occur at early age ever since the first day or a week of life, especially in vertical transmission. The consequences may depend on the virus strain and pathogenicity and the age of the birds first exposed to the virus (Smyth, 2017). The impact of the infection also depending on the viral load and the existence of maternal antibodies when the infection occurs. Besides, the presence of other enteric viruses or pathogens that may co-infected with such as avian nephritis virus (ANV), avian orthoreovirus (ARV) and fowl adenovirus (FAdV) may further enhance the pathogenicity of the virus.

The primary tissue tropism of the virus is in the intestine, kidney, liver, pancreas, and spleen, causing growth retardation, kidney disease and White Chicks hatchery disease.

2.4 Clinical Signs and Pathogenesis

Chicken astroviruses (CAstV) have been associated with mild gastroenteritis and growth depression in poultry, especially in chickens, turkeys, and ducks, and in some cases, it may lead to mortality (Sharma *et al.*, 2017). Recently, CAstV has also been implicated as the factor for “white chicks” condition related to increased embryo/chick mortality and weakness and white plumage of hatched chicks (Sajewicz-Krukowska *et al.*, 2016). According to Smyth (2017), CAstV is a possible pathogen for runting stunting syndrome (RSS) and severe kidney disease with visceral gout.

A runted chick appeared to hatch with a smaller size, whereas a stunted one will show delayed development and fail to grow normally. Generally, the disease is characterized with down and immature feathering, yellow coloration and small comb and beak. Usually, this condition occurs at the age of six to twelve days post-hatch, but it can be up to three week. CAstV group B can affect liver and kidneys, causing severe kidney disease with visceral gout outbreaks with 40% mortality in India (Bulbule *et al*., 2013). Poultry with white chick syndrome may hatch with pale plumage, weak, stunted, low survival rate, and typically same with RSS (Smyth, 2017).

Smyth *et al.* (2007) claimed that CAstV and other enteric viral infections might alter the normal intestinal environment, which can facilitate dysbacteriosis and imbalance of normal flora, frequently occurs during 20-30 days post-hatch causing poor digestibility performance and weakened intestinal barrier protection.

2.5 Transmission

The infection of CAstV occasionally occurs at an early age, and it can be transmitted horizontally via fecal-oral route or even vertically in some strains of CAstV derived from naïve in-lay parents, while the chicks are potentially shedding a high amount of virus (Smyth, 2017). In a quantitative molecular survey, the level of virus shedding by a chick that just hatched was found to be higher than the level of CAstV shedding from a chick that becomes infected horizontally later after hatch. This finding indicates the possibilities of vertical transmission since CAstV is one of the earliest viruses capable of infecting chicks (in the embryo) at the moment when immunity poorly develops (Smyth, 2017). In the Finnish breeder flock, the chicken seemed to be infected with the disease once in a lifetime upon vertical transmission, preventing further vertical transmission that may arise from acquired immunity.

2.6 Epidemiology and Risk Factor

Epidemiologically, chicken astrovirus (CAstV) infection has been reported throughout different geographical regions (Oluwayelu & Todd, 2012). Antibodies against CAstV also have been detected in the field serum of broiler chickens across the United Kingdom, the Netherlands, Spain, Australia, and the United States (Baxendale and Mebatsion, 2004). Besides, Pantin-Jackwood *et al.* (2006) also can detect CAstV from intestinal and fecal samples from both healthy and flock that are affected with enteritis and growth problems across the United States. Not only that, in the UK and Germany, Smyth *et al*. (2009) discover the presence of the virus in the cloacal swab and intestinal content of broiler chicken flock. However, the seroprevalence of the CAstV in commercial chickens in Malaysia has not been studied.

Two antigenically different CAstV, CAstV 1167 and CAstV 612 have been detected in broiler and parent flocks in the UK. These viruses were detected with lower levels in grandparent (GP) and great grandparent flocks and widespread among parent flocks throughout the European poultry farm.

In Bangladesh, Ali *et al.* (2020) discovered that seasons play an essential role in disease transmission when the prevalence reported in winter is higher than in summer. Besides, the virus can withstand high temperate up to 60 °C and even low pH conditions (Ali *et al.*, 2020).

2.7 Diagnosis

Diagnosis of CAstV infection could be made by accessing all the characteristics and clinical signs of the chickens associated with poor growth, enteritis and diarrhea, runting stunting syndrome, severe kidney disease of young broilers with visceral gout and the “White Chicks” hatchery disease. However, in some cases, chickens without any clinical signs might be tested positive. According to Ali *et al.* (2020), a further detailed study is needed to prove the association between infection and clinical disease. After all, characterization and identification of CAstV is of paramount importance since certain strains of CAstV are ubiquitous and can be detected from healthy chickens. Like other avian astrovirus members, CAstV can be diagnosed by virus isolation, electron microscopy, serologic methods, RT-PCR, and real-time RT-PCR (Long *et al.*, 2018).

Isolation and identification of the CAstV can be performed either in cell culture or fertilized embryonated chicken eggs. Isolation of CAstV in the SPF chicken embryonated eggs from White Chick Syndrome (WCS) chickens has shown to cause delayed hatch, embryo or chick mortality, weak chicks, subcutaneous edema of the head and neck, green coloring of the plumage, allantoic fluid, and yolk sac. In addition, the infected embryo may show organ lesions, including but not limited to enlarged pale livers with petechial hemorrhages (Sajewicz-Krukowska *et al.*, 2016) together with hemorrhage, edema, dwarfing, deformities, and a gelatinous appearance (Nu˜nez *et al*., 2016). The virus can also replicate in chick embryo liver cell (CEL) culture prepared from 14-day-old SPF embryo (Baxendale & Mebatsion, 2004) before it adapts to grow in LMH cell culture, a chicken hepatocellular carcinoma cell line (Long *et al*., 2018).

One of the principal approaches for diagnosing CAstV is electron microscopy, but this method depends solely on observing star-like morphology in the suspected sample (Madeley and Cosgrove, 1975). The reliability of electron microscopy for astroviruses can be increase by immune electron microscopy or immunofluorescence of astrovirus-infected cell cultures (Caul & Appleton, 1982; Reynolds & Saif, 1986).

Serological methods that can be used for detecting astrovirus includes virus neutralization and enzyme-linked immunosorbent assay (ELISA), while immunofluorescence test can be used to demonstrate the CAstV presence within fixed tissue and cell culture (Long *et al*., 2018). The assay has been used to detect CAstV612 or CAstV11672 infected cell cultures infected (Todd *et al.*, 2009). According to Baxendale and Mebatsion (2004), serum neutralisation test among field sera positive for CAstV revealed the presence of gel precipitating antibodies and suggested that the gel diffusion test may be of value as an inexpensive, technically simple flock test for CAstV. An indirect ELISA for the serological diagnosis of Group B CAstV has been developed and described (Skibinska *et al.*, 2015). Recently, Kang *et al*. (2012) showed that in situ hybridization technique is a reliable technique to demonstrate the replications of CAstV, ANV-1, and ANV-2 in lesions within fixed tissues.

A definitive diagnosis of CAstV can be made possible with the development of the nucleic acid-base test, including reverse transcriptase-polymerase chain reaction (RT-PCR) (Oluwayelu & Todd, 2012). RNA is extracted from the samples before underwent reverse transcription to obtain cDNA, specific for gene fragment from CAstV followed by PCR (Nu˜nez *et al.*, 2015).

2.8 Prevention and Control

Developing an effective strategy to combat pathogenic CAstV infection are challenging since no commercial or autogenous vaccines are readily available as astroviruses are difficult to replicate in quantities suitable for inactivated vaccine development (Long *et al*., 2018). Therefore, prevention and control measures can be done by implementing good hygiene and biosecurity since no effective treatments nor vaccines are available in the market (Reynolds and Schultz-Cherry, 2008). Hence, the solution to overcome chicken astrovirus infection is merely by minimizing the risk (Smyth, 2017).

Like other non-enveloped viruses, astrovirus is highly resistant to the harsh environment and commonly used disinfectants (Ali *et al*., 2020). A study by Schultz-Cherry *et al*. (2001) stated that the only disinfectants potent to kill the CAstV are 0.3% formaldehyde, 0.1% b-propiolactone, 1.5% Virkon S, and 90% methanol. The virus can be transmitted by darkling beetles where it can act as a vector (Rosenberger, 2010). In addition to the study by Smyth (2017), showed that CAstV could be detected by RT-PCR in the visceral tissue and washing from the surface of the beetles. Thus, according to Oluwayelu and Tood (2012), elimination of CAstV in the infected farm should include removing and disposing of all the fecal material in a process that will not contaminate the entrances of the houses with runoff followed by thorough disinfection.

2.9 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) test is a serological diagnostic method utilized to detect antigenic properties, primarily proteins including hormones, bacterial antigens and antibodies. There are several types of ELISA, namely indirect, direct competitive, antibody-sandwich, double antibody– sandwich, direct and indirect ELISA. Indirect ELISA is useful for screening specific antibodies in the serum, and the results are assessed with a spectrophotometer or spectrofluorometer. The measured reading is proportional to the amount of specific antibody in the serum tested. This study utilizes a 96-well pre-coated with inactivated CAstV Group B antigen, which will provide a quantitative result. The assay has been used to screen breeder flocks for seroconversion against CAstV B group strains only before or during lay. It can also be used in longitudinal serological surveys to determine CAstV B group seroconversion via the vertical transmission of the virus (Smyth 2017).

3.0 MATERIALS AND METHOD

3.1 Study design

A seroprevalence of chicken astrovirus (CAstV) in commercial broiler breeders in Peninsular Malaysia was carried out using archives serum samples that were submitted to the Avian Diagnostic Unit, Laboratory of Vaccine and Biomolecules, Institute of Bioscience, UPM. A total of 415 serum samples from broiler breeders of different ages ranging from 30 weeks-old to 62 weeks-old were considered in this study. These serum samples came from commercial breeder flocks in two different states in Peninsular Malaysia.

3.2 Serum samples

The archived serum samples were in good condition and well stored in -20^{o}C. All the serum samples were more than 50 µl and free from hemolysis. Before performing the assay, the serum samples were thawed at kept at 4^{o}C.

3.3 Enzyme-linked immunosorbent assay (ELISA)

The ELISA test, which is Chicken Astrovirus Group B Antibody test kit (CAstV Gp B) manufactured by BioCheck (UK) Ltd. was used to quantify the antibody levels against CAstV Gp B in the chicken's serum sample. The primary materials required to run the test includes serum, the 96-well microtitre plates (pre-coated with inactivated CAstV Gp B antigen), one known negative and positive control, conjugate reagent, substrate solution, stop solution, diluent reagent, washing buffer (phosphate-buffered saline) and microtitre plate reader with 405 nm filter.

Firstly, all the serum samples were diluted to 1:100 in sample diluent regent with a 2-step dilution procedure. 5µl of each serum sample was dispensed into a dilution plate before adding 245 µl of sample diluent reagent using a multichannel pipette to make it 1:50 dilution. Next, another set of dilution plate was prepared by loading 125 µl of sample diluent reagent followed by transferring 125 µl of diluted serum from the previous dilution, becoming 1:100 dilution factor.

The coated plate was labeled accordingly prior to the test procedure. 100 µl negative and positive control were added into wells A1-B1 and wells C1-D1, respectively. Well E1 and F1 were left as empty well. Then, 100 µl of diluted samples were added into the appropriate coated plate according to the label and covered with a lid before incubated for 30 minutes at room temperature (22-27 °C). All filled wells were aspirated to remove the contents and washed with the washing buffer three times. 200 µl of washing buffer was aspirated and refilled into the wells for the first wash. The wash solution was discarded. The subsequent wash was done with 250 µl of washing buffer. The plate was tapped on the absorbent paper/tissue paper to remove the remaining moisture.

After that, 100 µl of conjugate reagent was added to all the wells. The coated plate was once again covered with a lid and incubated at room temperature (22-27 °C) for 30 minutes. The washing procedures were repeated.

Following the second wash, 100 µl of substrate reagent was added into the wells, and the plate was incubated for 15 minutes at room temperature. After 15 minutes, a stop solution was added to each well to terminate the reaction. Colour changes from colourless to yellow will occurred. The plate was read by using Microtitre Plate Reader (BioTek® 800™ TS Absorbance Reader) at 405 nm within 30 minutes.

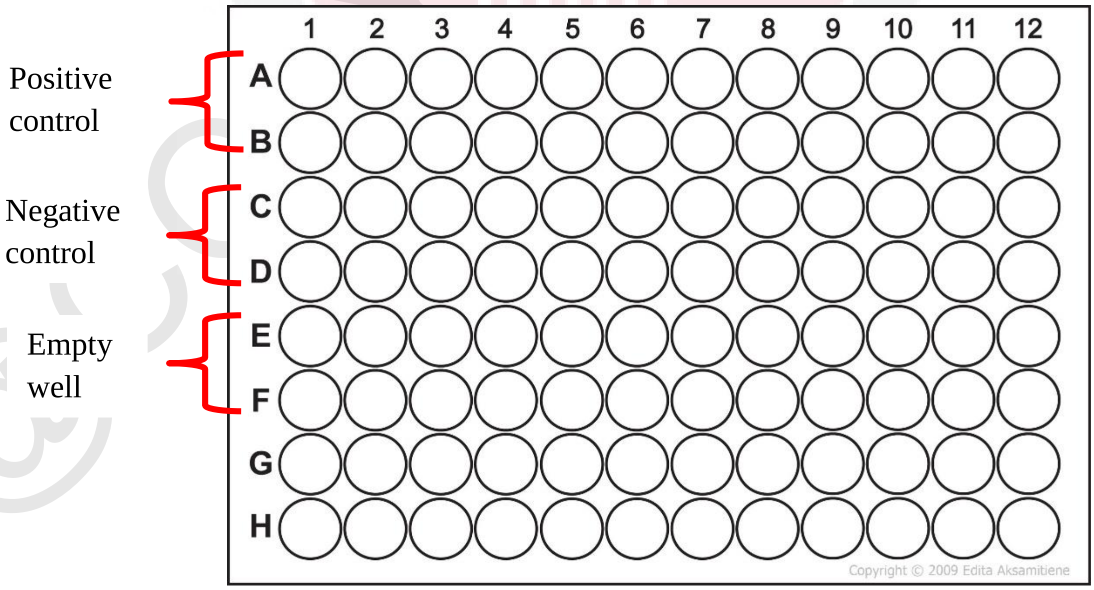

Figure 1: 96-well-plate illustration for CAstV ELISA Test

Each serum sample were run in duplicates. The average value from both tests was taken as the final value.

3.4 Result interpretation

The ELISA test is considered valid if the mean Negative Control absorbance is lower than 0.3. The difference between mean Negative Control to the mean Positive Control is higher than 0.15. For interpreting the result, the relative amount of antibodies is calculated by reference to the Positive Control, expressed as S/P ratio (Sample to Positive Ratio).

$$\frac{\text{Mean of test sample} - \text{Mean of negative control}}{\text{Mean of positive control} - \text{Mean of negative control}} = \text{S/P ratio}$$

The samples with S/P value equal to more than 0.7 are considered as positive antibody detection. The value of 0.5-0.699 and less than 0.5 are considered as suspect and no antibody detected, respectively. From the S/P value, antibody titer from the diluted serum sample (1:100 dilution) is calculated using the following formula:

$$\text{Log10 Titre} = 1.1 * \text{Log10 (S/P)} + 3.156$$

$$\text{Antilog} = \text{Titre}$$

3.5 Data analysis

All the data were evaluated in percentage calculation format and the mean values were used to calculate the seroprevalence of CAstV. The analysis of variance (ANOVA) method was used to determine the difference between the three different chickens' age groups and the levels of antibody titer against CAstV. The calculated P-value less than 0.05 is considered statistically significant.

4.0 RESULTS

A total of 415 serum samples from commercial broiler breeder chickens of different ages were used in this study to detect specific antibody titers against group B CAstV using an indirect ELISA kit. Among all of the samples tested, 404 samples were detected positive, and the remaining 11 samples were considered as suspected containing CAstV specific antibodies based on the ELISA kit instructions manual. None of the samples were tested negative (Figure 2). Since the suspect status can be considered positive as recommended by the manufacturer of the kit, therefore, all the 415 serum samples were considered positive for CAstV antibody (Table 1).

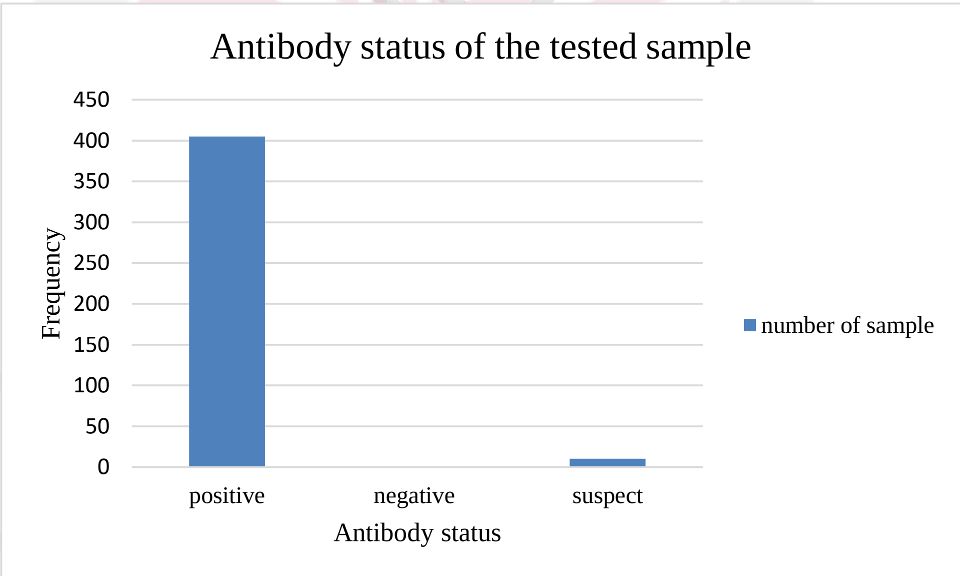

Figure 2: Graph showing the antibody status of broiler breeder chickens against group B CAstV

Table 1: Overall result for seroprevalence of CAstV in commercial broiler breeders

Total No. of sample	**Results**	
	No. of Positive	**No. of Negative**
415	415	0
	100%	0%

All the samples detected positive for antibody against CAstV were analysed statistically based on One-way ANOVA to determine any differences between the antibody titer and the chickens age groups. The serum samples were divided into three groups, Group 1, chickens that were less than 40 weeks of age, 40 to 50 weeks of age (group 2) and more than 50 weeks of age (group 3) (Table 2). The antibody titer values were converted into square root values to have normally distributed data.

Statistically, there was a significant difference ($p \leq 0.05$) in the antibody titers of chickens of age group 40 to 50 weeks compared to the chicken of age group less than 40 weeks of age ($p = 0.036$) and chicken of age group more than 50 weeks of age ($p = 0.021$). However, the antibody titer of chicken of the age group less than 40 weeks and more than 50 weeks were not significantly different ($p = 0.925$) (Table 3).

Table 2: The mean antibody titer for three different age groups

Group	Age	Antibody titer			
		Count	**Mean**	**Standard deviation**	**Standard error of mean**
1	< 40 weeks	120	3111.49	1505.17	137.40
2	40 to 50 weeks	150	2730.26	1202.70	98.20
3	> 50 weeks	145	3101.29	1395.35	115.88

Table 3: Post-Hoc test, LSD showing the difference of antibody titer between each age groups

(I) Age grouping			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	g1_<40 wks	g2_40-50wks	3.1645*	1.50099	**0.036**	0.2139	6.1150
		g3_>50wks	-0.1430	1.51245	0.925	-3.1160	2.8301
	g2_40-50wks	g1_<40wks	-3.1645*	1.50099	**0.036**	-6.1150	-0.2139
		g3_>50wks	-3.3075*	1.42729	**0.021**	-6.1131	-0.5018
	g3_>50 wks	g1_<40wks	0.1430	1.51245	0.925	-2.8301	3.1160
		g2_40-50wks	3.3075*	1.42729	**0.021**	0.5018	6.1131

* The mean difference is significant at the 0.05 level

5.0 DISCUSSION

Several studies have confirmed the detection of antibodies against chicken astrovirus (CAstV) in commercial chickens worldwide (Baxendale and Mebatsion, 2004). In line with that, a recent study confirmed the detection of CAstV in Malaysian poultry farms by RT-PCR (Raji *et al.*, 2019). In addition, sequencing confirmed the presence of Group B CAstV, which in line with the ELISA test kit that is used. Furthermore, a pathogenicity study confirmed that CAstV in Malaysia is pathogenic and can lead to clinical signs and lesions associated with an enteric infection where the viruses can be isolated from the inoculated and sentinel exposed specific-pathogen-free (SPF) chickens (Raji, 2021).

However, the seroprevalence of CAstV in commercial chickens, especially in broiler breeder flocks, is not known. In conjunction with that, this study aims to determine the seroprevalence of CAstV in selected commercial broiler breeder farms in Peninsular Malaysia. A commercially available indirect enzyme-linked immunosorbent assay (ELISA) that detects Group B CAstV was used to determine the antibody levels against CAstV.

The results showed that the seroprevalence of CAstV in broiler breeder chickens in the selected farms in Peninsular Malaysia is high, where all the tested samples were positive. As in the UK, a study shows that the prevalence among broiler parent flocks was at 53% (415/781) with the age range from 9 - 50 weeks old (Todd *et al.*, 2017). The significant difference between the prevalence of these two countries probably due to dissimilarities in the age range from the study where older birds were studied in Malaysia. Likewise, multiple age farming increases the risk from recovered aged hens that are not exhibiting clinical signs but could still be shedding the virus (Koci *et al*., 2000).

In addition, the high incidence of the serological prevalence is also probably associated with several factors, such as the virus's ability to withstand several disinfectants (Guy *et al*., 2009, Ali *et al*., 2020). Hence cleaning and biosecurity control measures are challenging. Furthermore, besides the horizontal transmission via the fecal-oral route, the virus can be transmitted vertically from an uncheck breeder flocks, increasing the chance of virus exposure to the susceptible birds (Smyth, 2017).

The potential of vertical transmission of CAstV has been recently reviewed by Todd *et al*. (2017) where the presence of CAstV was recorded in one-day-old chick and dead-in-shell chick, possibly transmitted from infected in-lay parents. Even though vertical and horizontal transmission can occur, there is no seroprevalence study comparing the antibody profiles of chicken infected via these two transmission routes. Besides, due to the practice of all-in all-out system, it will increase the possibility for all of the chickens been exposed to the virus.

Based on statistical analysis, the antibody titers of chicken at 40 to 50 weeks of age are statistically different ($p \leq 0.05$) than the group less than 40 weeks and more than 50 weeks of age. In contrast, there is no significant difference ($p>0.05$) between chicken at the age of less than 40 weeks and more than 50 weeks. The reduction of antibody titer among chickens 40 to 50 weeks of age compared to chicken less than 40 weeks of age might be influenced by decreasing in the susceptibility of the birds and/or lower levels of virus multiplication and excretion. As a result, the spreading of this virus is less efficient among older birds (Todd *et al*., 2017). Other than that, Todd *et al*. (2017) also stated that the astrovirus-specific antibody levels decrease with time as the chicken ages. However, the increase of antibody titer in the chicken more than 50 weeks of age possibly suggests the occurrence of re-infections among older birds even though this it is not common (Todd *et al*., 2017).

Although the broiler breeders are showing antibody titers against CAstV, based on the information from the farm manager, the production performances of the birds were within the normal range. No obvious clinical signs are mortality associated with CAstV infection such as enteritis and kidney disease associated with visceral gout were detected on the farm. Further study needs to be carried out to determine the impact of CAstV infection on broiler breeder birds. Nevertheless, the ELISA is suitable for monitoring flocks for seroconversion against the group B CAstV before laying period as a serological assessment in the potential transmission of the virus vertically to susceptible birds (Smyth, 2017). Once the flocks are confirmed free from CAstV, stringent biosecurity, enhanced timing, down-time between production cycles with adequate cleaning and decontamination of the area will further mitigate the chances of infection with avian astrovirus (Koci and Schultz-Cherry, 2002).

6.0 CONCLUSION & RECOMMENDATION

Based on this study, the seroprevalence of CAstV in selected broiler breeder farms in Peninsular Malaysia is high, reaching 100%. However, a further serological study involving younger broiler breeder flocks and the economic impact of the virus need to be carried out. Therefore, the study recommendation is to perform sampling both for serology and virus detection from birds of different ages, including day-old chicks, birds before and after laying. Besides, taking samples from other broiler breeder farms throughout Peninsular Malaysia will establish a better understanding of the seroepidemiology of CAstV infection. Other than that, research on viral detection and measurement of viral shedding in birds with different levels of antibody titers will provide valuable information on CAstV infection and immunity for viral clearance. In this study, limited history and data were obtained from the studied farms. The information such as biosecurity measures and farm management are essential to minimise the risk factors associated CAstV infection. All in all, this study has provided some insight and valuable information for further research towards developing proper prevention and control measures against CAstV infection in broiler breeder farms.

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APPENDICES

Sample (B7)	Average titer	Titer group	Result
1	6091.5	8	pos
2	2086.0	4	pos
3	4293.5	6	pos
4	5887.5	7	pos
5	5065.5	7	pos
6	3691.5	6	pos
7	1646.5	3	pos
8	2913.0	5	pos
9	1337.0	2	pos
10	5385.0	7	pos
11	9493.5	9	pos
12	3095.0	5	pos
13	5478.5	7	pos
14	2902.5	5	pos
15	3750.0	6	pos
16	5291.0	7	pos
17	1629.5	3	pos
18	4046.0	6	pos
19	3648.5	6	pos
20	5141.5	7	pos
21	2657.5	5	pos
22	4985.0	7	pos
23	2879.5	5	pos
24	5601.0	7	pos
25	6119.0	8	pos
26	3372.0	5	pos
27	4618.5	7	pos
28	3568.0	6	pos
29	7070.0	8	pos
30	1670.0	3	pos

Sample (B1)	Average titer	Titer group	Result
1	2435.5	4	pos
2	2456.0	4	pos
3	2342.5	4	pos
4	2451.5	4	pos
5	1274.0	2	pos
6	1195.5	2	pos
7	1255.5	2	pos
8	1383.0	2	pos
9	3655.5	6	pos
10	677.0	1	sus
11	2213.5	4	pos
12	1661.0	3	pos
13	3311.0	5	pos
14	2558.0	5	pos
15	3346.5	5	pos
16	5267.0	7	pos
17	3786.5	6	pos
18	1475.5	2	pos
19	2158.5	4	pos
20	1796.0	3	pos
21	2207.5	4	pos
22	1472.0	2	pos
23	1093.0	2	pos
24	3276.5	5	pos
25	2353.5	4	pos
26	1267.0	2	pos
27	1589.5	3	pos
28	1595.0	3	pos
29	1777.5	3	pos
30	3805.5	6	pos

Sample (C4)	Average titer	Titer group	Result
1	874.0	1	sus
2	3717.5	6	pos
3	2084.5	4	pos
4	911.5	1	sus
5	2449.5	4	pos
6	1682.5	3	pos
7	1899.5	3	pos
8	2616.0	5	pos
9	4345.0	6	pos
10	3552.0	5	pos
11	968.5	2	pos
12	1988.5	3	pos
13	1895.0	3	pos
14	4990.0	7	pos
15	1954.5	3	pos
16	3174.0	5	pos
17	4912.0	7	pos
18	3676.5	6	pos
19	3562.0	6	pos
20	1621.0	3	pos
21	5048.5	7	pos
22	1359.5	2	pos
23	3859.5	6	pos
24	2789.5	5	pos
25	3533.0	6	pos
26	2143.0	4	pos
27	881.5	1	sus
28	2062.5	4	pos
29	3191.5	5	pos
30	1890.5	3	pos

Sample (C2)	Average titer	Titer group	Result
1	3683.5	6	pos
2	3872.0	6	pos
3	2624.0	5	pos
4	3425.0	5	pos
5	4948.5	7	pos
6	3318.0	5	pos
7	4707.0	7	pos
8	5128.0	7	pos
9	3450.0	5	pos
10	3423.0	5	pos
11	3079.5	5	pos
12	1749.5	3	pos
13	1716.0	3	pos
14	2629.0	5	pos
15	2621.0	5	pos
16	2716.5	5	pos
17	2051.5	4	pos
18	4989.0	7	pos
19	3640.5	6	pos
20	4527.5	7	pos
21	2537.5	5	pos
22	3689.0	6	pos
23	1799.0	3	pos
24	4801.5	7	pos
25	4905.0	7	pos
26	2277.0	4	pos
27	3401.5	5	pos
28	3438.5	5	pos
29	2905.5	5	pos
30	3142.0	5	pos

Sample (A7)	Average titer	Titer group	Result
1	3311.5	5	pos
2	1185.5	2	pos
3	1588.0	3	pos
4	1200.5	2	pos
5	1731.0	3	pos
6	2484.5	4	pos
7	1613.0	3	pos
8	1008.5	2	pos
9	1307.5	2	pos
10	1267.5	2	pos
11	1128.0	2	pos
12	1748.5	3	pos
13	2572.0	5	pos
14	3834.5	6	pos
15	1129.5	2	pos
16	2617.0	5	pos
17	2140.5	4	pos
18	2517.5	5	pos
19	2224.5	4	pos
20	1322.0	2	pos
21	3410.5	5	pos
22	2298.5	4	pos
23	4238.0	6	pos
24	2332.5	4	pos
25	1348.5	2	pos
26	2761.5	5	pos
27	1697.0	3	pos
28	1229.0	2	pos
29	1774.5	3	pos
30	1024.0	2	pos

Sample (A5)	Average titer	Titer group	Result
1	3097.0	5	pos
2	3638.0	6	pos
3	2721.5	5	pos
4	3618.0	6	pos
5	4103.5	6	pos
6	4162.0	6	pos
7	7074.0	8	pos
8	5982.5	7	pos
9	2673.0	5	pos
10	2229.5	4	pos
11	2972.5	5	pos
12	1903.0	3	pos
13	1999.0	3	pos
14	2197.0	4	pos
15	2538.0	5	pos
16	1960.0	3	pos
17	2090.5	4	pos
18	5417.5	7	pos
19	1436.5	2	pos
20	1700.5	3	pos
21	2505.0	5	pos
22	2705.0	5	pos
23	2349.0	4	pos
24	1911.5	3	pos
25	997.5	2	pos
26	3655.5	6	pos
27	1538.0	3	pos
28	1286.0	2	pos
29	3404.0	5	pos
30	2272.5	4	pos

Sample (A3)	Average titer	Titer group	Result
1	4440.0	6	pos
2	4426.0	6	pos
3	2474.0	4	pos
4	2135.0	4	pos
5	4483.5	6	pos
6	4398.5	6	pos
7	3931.0	6	pos
8	3123.0	5	pos
9	2049.0	4	pos
10	2413.5	4	pos
11	2477.0	4	pos
12	4390.0	6	pos
13	4168.0	6	pos
14	4371.0	6	pos
15	2947.5	5	pos
16	2057.0	4	pos
17	4125.5	6	pos
18	2305.0	4	pos
19	2902.5	5	pos
20	4644.5	7	pos
21	2611.5	5	pos
22	5048.5	7	pos
23	2240.5	4	pos
24	2125.0	4	pos
25	2691.5	5	pos
26	3783.0	6	pos
27	2524.5	5	pos
28	2031.5	4	pos
29	2568.0	5	pos
30	2637.5	5	pos

Sample (T11)	Average titer	Titer group	Result
1	1641.0	3	pos
2	2221.0	4	pos
3	1665.5	3	pos
4	4029.5	6	pos
5	2184.5	4	pos
6	4569.5	7	pos
7	1772.5	3	pos
8	764.5	1	sus
9	1895.0	3	pos
10	3982.5	6	pos
11	5768.0	7	pos
12	5344.5	7	pos
13	2424.5	4	pos
14	2586.5	5	pos
15	4434.5	6	pos
16	2155.0	4	pos
17	726.0	1	sus
18	2436.0	4	pos
19	4001.5	6	pos
20	1862.5	3	pos
21	1604.5	3	pos
22	2286.5	4	pos
23	3655.5	6	pos
24	3242.5	5	pos
25	4994.0	7	pos
26	4807.0	7	pos
27	1060.0	2	pos
28	2637.5	5	pos
29	3076.0	5	pos
30	1958.0	3	pos

Sample (T12)	Average titer	Titer group	Result
1	2021.5	4	pos
2	2598.0	5	pos
3	2833.5	5	pos
4	2366.0	4	pos
5	4433.5	6	pos
6	4109.0	6	pos
7	1531.0	3	pos
8	3897.5	6	pos
9	1265.5	2	pos
10	2553.5	5	pos
11	1675.5	3	pos
12	4599.5	7	pos
13	1174.0	2	pos
14	1608.0	3	pos
15	3095.5	5	pos
16	2198.5	4	pos
17	2703.0	5	pos
18	2641.0	5	pos
19	3443.5	5	pos
20	2765.0	5	pos
21	3221.0	5	pos
22	2458.0	4	pos
23	4058.0	6	pos
24	3677.5	6	pos
25	1692.0	3	pos
26	3640.0	6	pos
27	3634.5	6	pos
28	1476.5	2	pos
29	1934.0	3	pos
30	1742.5	3	pos

Sample (T9)	Average titer	Titer group	Result
1	3200.5	5	pos
2	2029.5	4	pos
3	1659.0	3	pos
4	2668.5	5	pos
5	3120.0	5	pos
6	3000.0	5	pos
7	3097.5	5	pos
8	1347.0	2	pos
9	2960.0	5	pos
10	3235.5	5	pos
11	3859.0	6	pos
12	4244.0	6	pos
13	1182.0	2	pos
14	1929.5	3	pos
15	3547.0	6	pos
16	977.5	2	pos
17	3253.5	5	pos
18	2242.5	4	pos
19	1857.0	3	pos
20	844.5	1	sus
21	1896.0	3	pos
22	1912.5	3	pos
23	3731.5	6	pos
24	1062.5	2	pos
25	3900.0	6	pos
26	1795.0	3	pos
27	3926.5	6	pos
28	2836.5	5	pos
29	1992.0	3	pos
30	3054.5	5	pos

Sample (T7)	Average titer	Titer group	Result
1	2537.0	5	pos
2	2595.5	5	pos
3	3970.0	6	pos
4	3857.0	6	pos
5	3748.0	6	pos
6	1989.0	3	pos
7	2867.0	5	pos
8	3156.5	5	pos
9	2209.5	4	pos
10	1581.5	3	pos
11	1126.5	2	pos
12	2059.0	4	pos
13	3000.5	5	pos
14	3338.0	5	pos
15	3052.5	5	pos
16	907.0	1	sus
17	4867.5	7	pos
18	2566.0	5	pos
19	1059.5	2	pos
20	4810.0	7	pos
21	3515.0	6	pos
22	1113.0	2	pos
23	3573.0	6	pos
24	4687.0	7	pos
25	4013.5	6	pos
26	4034.0	6	pos
27	909.5	1	sus
28	3746.0	6	pos
29	3041.5	5	pos
30	3107.5	5	pos

Sample (T5)	Average titer	Titer group	Result
1	9294.5	9	pos
2	2985.5	5	pos
3	2400.0	4	pos
4	2782.0	5	pos
5	5301.5	7	pos
6	2776.0	5	pos
7	8229.0	9	pos
8	2173.5	4	pos
9	2324.5	4	pos
10	2056.5	4	pos
11	3158.5	5	pos
12	1758.5	3	pos
13	5476.0	7	pos
14	3110.0	5	pos
15	3051.5	5	pos
16	1335.5	2	pos
17	2601.5	5	pos
18	3008.5	5	pos
19	3806.0	6	pos
20	1928.0	3	pos
21	3047.0	5	pos
22	2666.5	5	pos
23	2012.5	4	pos
24	3895.5	6	pos
25	3470.0	5	pos
26	2183.5	4	pos
27	4231.5	6	pos
28	3760.5	6	pos
29	3327.5	5	pos

Sample (T2)	Average titer	Titer group	Result
1	3732.5	6	pos
2	817.0	1	sus
3	2609.5	5	pos
4	2742.0	5	pos
5	2914.5	5	pos
6	4815.0	7	pos
7	4039.0	6	pos
8	4389.5	6	pos
9	4854.0	7	pos
10	3029.5	5	pos
11	3847.5	6	pos
12	4780.5	7	pos
13	4122.5	6	pos
14	4222.0	6	pos
15	4133.0	6	pos
16	2031.0	4	pos
17	3992.0	6	pos
18	4553.0	7	pos
19	2240.5	4	pos
20	2270.5	4	pos
21	1629.5	3	pos
22	1806.0	3	pos
23	3006.0	5	pos
24	2916.0	5	pos
25	1868.5	3	pos
26	1894.0	3	pos
27	1687.5	3	pos
28	3087.5	5	pos

Sample (T1)	Average titer	Titer group	Result
1	3540.0	6	pos
2	4202.5	6	pos
3	3167.0	5	pos
4	2862.5	5	pos
5	6286.5	8	pos
6	5466.5	7	pos
7	6282.0	8	pos
8	1859.5	3	pos
9	1484.0	2	pos
10	4308.5	6	pos
11	1998.0	3	pos
12	2387.5	4	pos
13	1613.0	3	pos
14	4564.0	7	pos
15	3328.5	5	pos
16	7109.5	8	pos
17	1157.0	2	pos
18	3596.0	6	pos
19	3111.0	5	pos
20	1347.0	2	pos
21	4783.0	7	pos
22	4568.5	7	pos
23	2689.5	5	pos
24	4255.0	6	pos
25	5374.5	7	pos
26	4059.0	6	pos
27	1927.5	3	pos
28	2779.0	5	pos

Category	Flock	Age (weeks)	Number of sample	Positive	Suspect	Mean titer	Standard Deviation	Standard Error
group 1 (<40wks)	B7	30	30	30	0	4180.4500	1835.7976	335.1692
	B1	33	30	29	1	2237.8833	1032.4026	188.4901
	C4	35	30	27	3	2654.4333	1249.4492	228.1172
	C2	37	30	30	0	3373.1833	1020.2000	186.2622
group 2 (40-50wks)	A7	40	30	30	0	2001.5167	870.8398	158.9929
	A5	42	30	30	0	2871.2500	1392.5765	254.2485
	A3	45	30	30	0	3217.4333	994.5680	181.5824
	T11	48	30	28	2	2859.5333	1388.5353	253.5107
	T12	49	30	29	1	2701.5500	991.7002	181.0589
group 3 (>50wks)	T9	54	30	29	1	2545.3667	983.9481	179.6435
	T7	56	30	28	2	2901.2500	1169.5701	213.5333
	T5	58	29	29	0	3384.5345	1771.5989	328.9777
	T2	60	28	27	1	3143.9286	1148.3502	217.0178
	T1	62	28	28	0	3575.2321	1610.1287	304.2857