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Antigenic Relatedness between a Classic Strain and Very Virulent Strain of Infectious Bursal Disease

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ABSTRACT

Gumboro disease or infectious bursal disease (IBD), caused by IBDV agents, leads to significant economic losses due to its acute and contagious nature, affecting chickens aged three to six weeks, resulting in immunosuppression with high morbidity and mortality rates. Despite preventive measures, including vaccination, outbreaks of IBD still occur. Variations in antigenic relatedness between vaccine strains and field viruses can affect vaccination efficacy. This study aimed to assess the antigenic relatedness between the Lukert strain and vvIBD through homologous and heterologous cross-neutralization tests using chicken embryo fibroblast (CEF) cultures. Twenty-five serum samples from five chicken groups were tested. The control group remained unvaccinated, while the first and second groups received a single dose of the Lukert strain or vvIBD vaccine at two weeks of age. The third and fourth groups were revaccinated twice with the Lukert strain or vvIBD vaccine. Neutralization test results and antigenic relatedness was determined using the Archeti and Horsfall formula. Virus re-identification was performed using PCR and sequencing. The neutralization test revealed an antigenic relatedness of 11.14% between the Lukert and vvIBD, indicating they belong to the same serotype with major differences in subtypes. Consequently, there is a low indication of cross-protection between the two strains. There was a significant ($P<0.05$) difference between the test group of viruses and serum sample antibodies. Given the low antigenic relationship, reference is needed for the development of vaccines homologous to field virus strains.

Key words: Antigenic relatedness; Cross-neutralization test; Infectious Bursal Disease.; Lukert strain; vvIBD strain

INTRODUCTION

Gumboro disease or infectious bursal disease is an acute contagious viral disease, immunosuppressive that affects chickens aged three to six weeks (Kurukulsuriya et al. 2016). The first outbreak was reported in Gumboro, Delaware in 1957 (Sali 2019). The virus belongs to the family *Birnaviridae* and genus *Avibirnavirus*. The virus has a high mutation rate, is environmentally resistant, non-envelope, double-stranded RNA (dsRNA), and bi-segmented (segment A and segment B) make the virus high error rate of viral RNA-dependent RNA polymerase (RdRp) leading the IBD virus naturally prone to varying degrees of genomic mutation or recombination that is leading the emergence of new mutant or recombinant strains in chicken (Jackwood et al. 2016; Wang et al. 2022). The IBD virus has two serotypes, which are serotype 1, a pathogenic virus in chickens, and serotype 2, a non-pathogenic virus to chickens, that isolated from turkeys (Workineh et al. 2022). Serotype 1 is classified based on pathogenicity and antigenicity into classical virulent (cv), variant, attenuated and very virulent (vv) IBDV (Van den Berg et al. 2004; Hayajneh and Araj 2023).

It was reported that poultry infected with the IBD virus will have a high mortality rate, atrophy of the bursa Fabricius, increased feed conversion ratio (FCR), and decreased meat production (Zachar et al. 2016). It impacts the poultry industry, causing most economic losses directly through imposed by immunosuppression in affected animals, can lead to secondary pathogen infections and affecting the efficacy of other vaccines (Gao et al. 2023). Several cases of acute IBD disease with high mortality have

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been reported in Indonesia, with broiler mortality reaching more than 25% and layer mortality more than 60% in un-vaccinated flocks (Wibowo et al. 2017).

The antigenic relatedness between strains of the virus can be determined by the neutralization test based on the R value (relatedness value) using the Archetti and Horsfall (1950) formula. The antigenic relatedness by the neutralization test, also illustrates cross-protection (OIE, 2018). Antigenic differences between strains in the field and commonly used vaccine strains cause the vaccine used to be only partially effective (He et al. 2019). The last few reports related to the global epidemiological situation regarding Gumboro disease are characterized by the emergence of novel antigenic variant, mosaic or distinct (Pikuła et al. 2018; Fan et al. 2019; Tomás et al. 2019).

A principal component of control and prevention is vaccination accompanied with proper biosecurity and maintenance management. The management of IBD in Indonesia is mostly dependent on vaccination using inactivated and live attenuated vaccine are commonly used to control IBD even in another country too (Müller et al. 2012). Even though preventative by use vaccinations and biosecurity measures have been implemented globally, the virus remains endemic around the world (Dey et al. 2019; Zaheer et al. 2022; Du et al. 2023). Therefore, there is a need to continuously assess and improve the vaccination program. The IBD virus has a rapid mutation rate that contributed to the emergence of mutant virus strains with different antigenicity compared to current commercial vaccinations (Hou et al. 2022). The reaction between classical and vvIBD strains will impact vaccine effectiveness in the field. In this study, the antigenic relatedness between the classical strains and vvIBD strains was determined through homologous and heterologous cross-neutralization tests using chicken embryo fibroblast (CEF) cultures.

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Committee of the Faculty of Veterinary Medicine at Udayana University approved this research procedure with Approval number B/119/UN14.2.9/PT.01.04/2024.

Materials

Five groups of twenty-five SPF chicken serum samples in total were used. The control group remained unvaccinated, and the first group is the serum of two-week-old SPF chickens vaccinated and received a single dose of Lukert strain intranasally, while the second group received the vvIBD strain. The third and fourth groups are the serum of four-week-old SPF chickens that were revaccinated twice with Lukert or vvIBD virus strains. Post three weeks of vaccine, samples of serum were collected for serum neutralization tests (SNT). The results of antisera titers in the third and fourth groups were analyzed for Relatedness value (R-value) described using the Archeti and Horsfall formula.

Cell culture

Chicken embryo-fibroblasts (CEF) cultures were prepared using eight to ten days old SPF chicken egg embryos. The tissue was dissembled in a petri dish and then rinsed with phosphate buffer saline (PBS). Subsequently, trypsinized (0.25% trypsin) at 37ºC for 5min. Then, filters, the cell was suspended in growth media and centrifuged and re-suspension in growth media containing Eagle's Minimum Essential Media (MEM) until the final pH was between 7.0 and 7.2. The cell content was $1.2x10^6$ cells/mL. The cell suspension was distributed on a microplate 100µL per well. Then incubated in a 5% CO_2 incubator at 37ºC for 1-3 days.

Serum neutralization test

The serum was in-activated at 56°C for 30min and make serial dilution in each well with Eagle's MEM. After each serum dilution, 100 $TCID_{50}$/50µL of virus was inoculated into all wells. The virus-serum incubated then inoculated into a cell monolayer. After that, 100µL of growth media was add then incubation in 5% CO_2 for 3-5 days and observed daily using an inverted microscope for the presence of cytopathic effect (CPE). Neutralization serum antibody titers were calculated based on the inverse of the highest serum dilution to the presence of CPE.

Polymerase chain reaction test and sequencing

PCR using SuperScript^r from Invitrogen. The forward primer used was IBD-F (5'- TCACCGTCCTCAGCTTAC -3') and the backward primer used was IBD-R (5'- TCAGGATTTGGGATCAGC -3'). PCR cycle stages are reverse transcription using same instruction from Omer and Khalafalla (2022). The electrophoresis uses 1% agarose. Sequencing using the Big Dye Terminator from Applied Biosystems®. The results were analyzed with MEGA v.6 software using BLAST search in GenBank.

Antigenic relatedness

This criterion is used to classify the antigenic relatedness of each virus into serotypes and subtypes based on neutralizing antibody titers. Neutralization antibody titers against different viruses are analyzed using the Archetti and Horsfall (1950):

$$R = \sqrt{Rx \times Ry}$$

In which the ratio Rx is the heterologous titer of virus x using antigen y divided by the homologous titer of virus x, and Ry is the heterologous titer of virus y using antigen x divided by the homologous titer of virus y. The Relatedness value between 0 to 0.10 indicated the difference in serotype, while 0.11 to 0.32 indicated a major difference in subtype, 0.33 to 0.70 indicate a minor difference, and over 0.70 to 1.00 indicate a little or no difference (He et al. 2014).

Data analysis

A one-way ANOVA was used to evaluate the statistical significance of the differences among the different groups. A $P<0.05$ was considered statistically significant.

RESULTS

The cross-neutralization tests were conducted homologous and heterologous on the samples of the control group, the first group (vaccine strain Lukert) and the

second group (vaccine strain vvIBD). The average neutralization antibody titer is presented in Table 1.

Table 1: The averages of neutralization antibody titers

Groups	$\bar{x}$ ±SD
Lukert virus – Lukert antibody	70.40±35.05[b]
Lukert virus – vvIBD antibody	8.00±0.00[a]
vvIBD virus – vvIBD antibody	35.20±17.52[a]
vvIBD virus – Lukert antibody	11.20±31.35[a]

Superscripts with different letters indicate significant differences.

Based on the Analysis of Variance (ANOVA), there were significant differences between virus test groups and serum sample antibodies with a significance of 0.043 ($P<0.05$). The interpretation of antigenic relatedness (R) based on Archetti and Horsfall's (1950) formula from antisera in the third and fourth groups is illustrated in Table 2. It described that the classic strain (Lukert) and vvIBD had an R-value of 0.1114, it is indicated that the two strains are in the same serotype with major subtype differences. Therefore, the antigenic relationship between the classical strain and vvIBD is 11.14%.

Table 2: Antigenic relatedness between Lukert and vvIBD

Anti-sera	Lukert	vvIBD
Lukert	1.00	
vvIBD	0.1114±0.08	1.00

Virus reidentification was conducted to verify that the virus used for inoculation in the virus neutralization test was the intended virus. The molecular detection using PCR identified an amplification at 479bp of the genomic fragment of the IBD virus. The electrophoresis results are presented in Fig. 1. The results of sequencing and BLAST were revealed that Lukert isolates belong to geno group A1 (classical), and vvIBD isolates belong to geno group A3 (vvIBD) based on Islam et al. (2021). A picture of the phylogenetic tree is presented in Fig. 2.

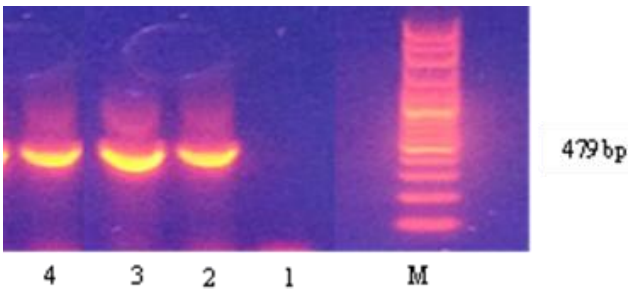

Fig. 1: Amplification results of the VP2 gene. M: Molecular size marker, (-): Negative Control, (+): Positive Control, 1: Lukert IBD sample, 2: vvIBD sample. Amplicons di electrophoresis 1% gel agarose.

DISCUSSION

Antigenic relatedness (R-value) is determined using a cross-neutralization test. Table 2 presents the R-value of 0.1114 (11.14%) between the Lukert strain and vvIBD. Therefore, it can be interpreted in serology with the neutralization test that the two strains are in the same serotype with major subtype differences (R-value ≥0.11). R values close to 1 (homologous) indicate values of almost 100% antigenic similarity (Cubas-Gaona et al. 2023). The antigenic similarity between the classical strain (Lukert) and vvIBD is only 11.14%. The poor antigenic relatedness between these two strains will reduce vaccine effectiveness in the field if there are differences between the virus in the field and the vaccine strain. He et al. (2019) described antigenic diversity in IBD serotype-1 field viruses in Southern China, that there are differences between field strains and used vaccine strains could indicate that used vaccine is partially effective. OIE (2018) indicated that the value of antigenic relatedness between strains can describe cross-protection based on neutralization tests. However, to confirm the potential ability of cross-protection, it is necessary to conduct a challenge test to ensure the protectivity of strains in vaccines.

Since the first report of Gumboro in 1957 (Eterradossi and Saif 2013), the disease has spread widely to various countries including Indonesia. Although IBDV can be control with implementation of vaccination use live attenuated or inactive vaccine, and adequate management, outbreaks of IBD is still continue reported (Li et al. 2015). The same statement from Wibowo et al. (2017), that with vaccination programs, IBD cases can be controlled but there are still reports of about 5-10% chicken mortality in Indonesia. In Sudan in 2022, a high mortality rate of IBD in vaccinated chickens of almost 51% was reported, which was thought to have occurred due to the emergence of a strain of vvIBD that was antigenically different from the virus in the vaccine used (Omer and Khalafalla 2022). Reassortment has been published over the past few years (Jackwood et al. 2016; Felice et al. 2017; Abed et al. 2018; He et al. 2019), resulting escape of IBDV strains from vaccination programs (Fan et al. 2019). The novel reassortant strains of IBD virus can contain a unique genetic background composition that can make two main phenotype for IBDV is immune-escaping strains from the selection on segment A or strains with functional advantages from the selection on segment B (Pikuła et al. 2021). There's have been report of novel variant IBD virus (Genotype A2dB1b) that responsible for high mortality and typical sign and lesion, indicate protection conferred by available commercial vaccines appears suboptimal (Legnardi et al. 2023).

The reassortment of IBDV with different pathogenicity and antigenicity on the farm has been observed in vivo by Chen et al. (2018). This is due to that IBD viruses are RNA viruses that have a high mutation rate, which can lead to modified antigenicity. The role of the A segment and B segment of the genome has contributed to the virulence of the IBD virus which affects the pathogenicity of the virus strain (He et al. 2019). The mutation of VP2 at position 222 from Proline in classic strain to Alanine in very virulent strain will determine differences in antigenic relatedness between these viruses (Jackwood and Sommer-Wagner 2011). The hypervariable region of VP2 (VP2 HVR) extending from residues 206 to 350 in the polyprotein is a major antigenic determinant that induces neutralizing antibodies (Dey et al. 2019). This protein is located in the capsid domain of the projection (P) part with four loops, each of which contains a neutralization epitope and is a site of frequent non-synonymous mutations (Eterradossi and Saif 2020). In

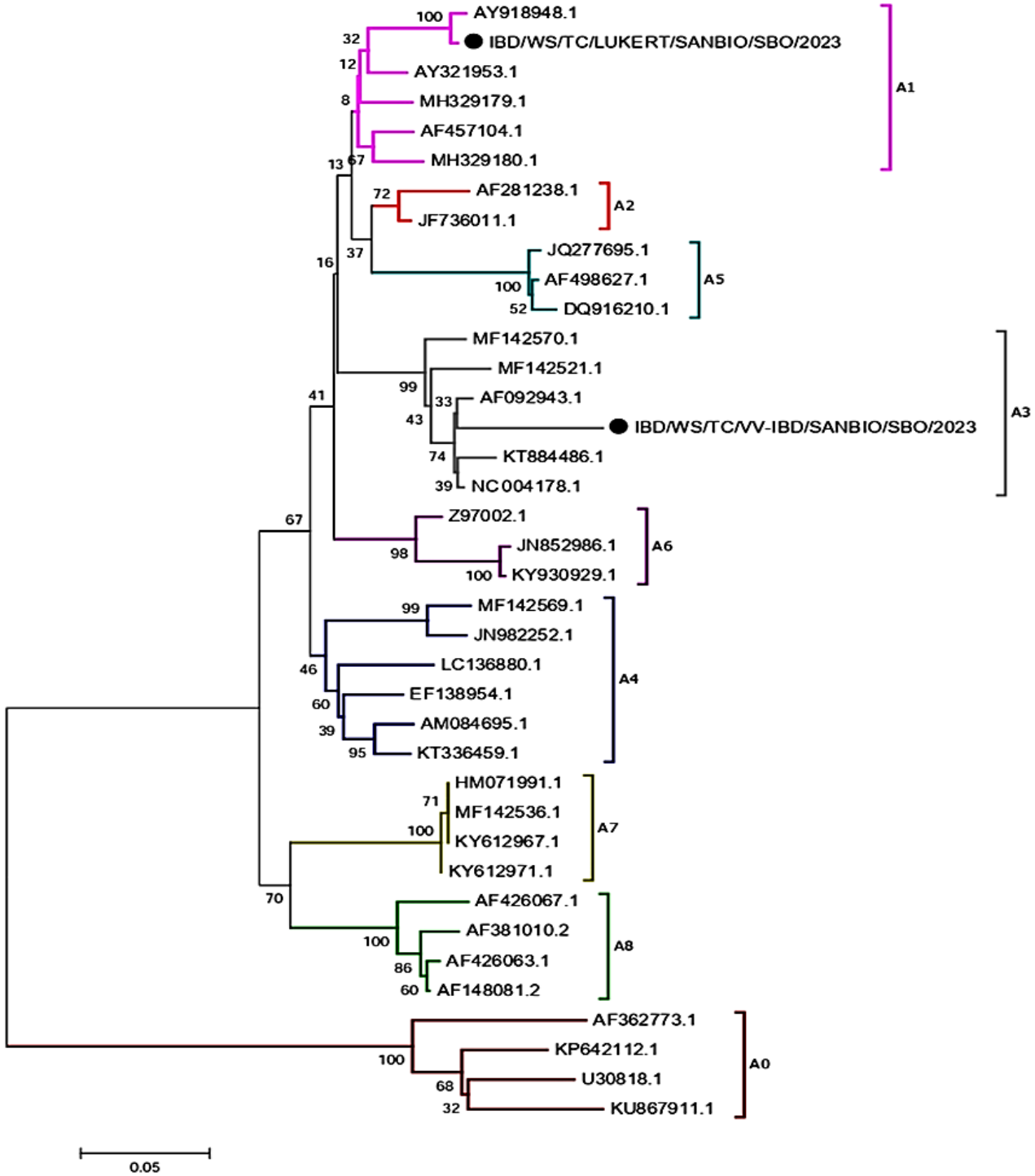

Fig. 2: Phylogenetic tree of vvIBD and Lukert strain. The virus isolates analyzed in this study are shown with a round mark (•).

general, the four amino acid residues identified as pathogenicity markers of IBD viruses, which are 222(A), 242(I), 256(I), 294(I), and 299(N), are reported to be conserved among vvIBD viruses, while those occupied by 222(P), 242(V), 256(V), and 299(N) are characteristic of classic strain IBD viruses (Kim et al. 2010), and it was recently shown that mutations in residues 318 and 323 of VP2 significantly affect neutralization (Fan et al. 2022).

According to several reports, the identification of different antigenic subtypes of IBDV strains in the field may be one of the major causes for the persistent and sporadic outbreaks of IBDV. Michel and Jackwood (2017) revealed antigenic drift that occurs in some strains in the field due to antigenic current vaccination with the same live vaccine strain, causing the virus in the field evolving and changing. This decrease the protection provided by live vaccinations regularly administered, which leads to IBD outbreaks that are still often reported in vaccinated farms. Dey et al. (2019) describe recent vaccine developments to control IBDV such as subunit vaccines, virus-like particle (VLP), DNA vaccines, and immune-complex vaccines. Although several types of vaccines have been produced for IBDV, presently remains a high demand for innovative, effective vaccines. Emerging variant strain could indicating that present vaccines are insufficient for controlling outbreaks caused by such strains (Jackwood et

al. 2016; Kurukulsuriya et al. 2016). Reverse genetic techniques are being used to construct different strains with high potential as vaccine candidate such as by inserting VP2 sequences into the backbone of vaccine strains, it has generated several chimeric viruses that can effectively protect chicken flocks (Gao et al. 2011; Shah et al. 2022). Attenuated IBDV, created by lowering VP1's RNA polymerase activity, can elicit immunological protection (Yang et al. 2020). The vaccination program must also be accompanied by proper biosecurity and maintenance management to reduce losses due to IBDV infection.

Conclusion

This study concluded that the antigenic relatedness between the classical strain (Lukert) and vvIBD was 11.14%, concluding that the two strains are under the same serotype with major subtype differences. As a result, it is critical to produce an effective vaccination that is appropriate for the strains in the field.

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Author's contribution

All authors were actively involved with different responsibilities. Derisna Sawitri Ungsyani and Arini Nurhandayani prepared a research proposal, and conceptualization, and conducted laboratory work. Derisna Sawitri Ungsyani, Gusti Ayu Yuniati Kencana, and I Nyoman Suartha: statistical analyses and write the manuscript. Tri Komala Sari, Ida Bagus Kade Suardana, and Tjok Gde Oka Pemayun: review of the final manuscript.

Conflict of interest

The authors declare there is no conflict of interest.

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