Short Communication

Detection of RHDV Antigen Using DAS ELISA Technique and the Influence of Breed, Sex, Age on the Incidence of RHDV Seroconversion in Asymptomatic Rabbits in West Java, Indonesia

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Abstract | The first case reported a seroprevalence study was conducted in the West Bandung District of West Java Province, Indonesia, revealed the presence of RHDV antibody in rabbit serums without clinical symptoms in some rabbit farms. The presence of antibodies to RHDV in rabbits without vaccination indicates exposure to the virus. Thus, this study aims to investigate the presence of RHDV antigen in asymptomatic seroconverted RHDV rabbits in West Bandung District, West Java Province, Indonesia also to see the relevance of seroconversion within rabbit's profile. Liver, intestines, and faeces were collected purposively from seroconverted rabbits which showed no clinical signs of RHDV. Samples assayed with commercial ELISA Kit using DAS Technique. The informations (age, sex, breed) of antibody titers against RHDV from previous study is used for samples profiling. The results showed all samples negative for RHDV antigen with average value ±0.04 (positive cut off >0.334). Seroconversion of RHDV from all seven villages where most seroconversion are showed by samples from young rabbits compare to adult rabbits, female rabbits compare to male rabbits but insignificant among breeds group of *Oryctolagus cuniculus* species or European rabbits. There were no RHDV antigen can be found from asymptomatic seroconverted RHDV rabbits in this study using DAS ELISA while seroconversion was affected by age and sex while there was no significant difference of seroconversion among breeds of *Oryctolagus cuniculus* or European rabbits which spreading among asymptomatic rabbits in seven West Bandung Regency villages, Indonesia.

Keywords | Rabbit haemorrhagic disease, Seroconversion, Asymptomatic, DAS ELISA

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INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an infectious disease of rabbits caused by the RHD virus (RHDV) of the genus *Lagovirus*, family *Caliciviridae*. The disease was first reported in China in 1984 which died within 1 to 2 days after the onset with mortality rate as high as 90% and the only symptom could be observed before death was

a slight fever (Liu et al., 1984) and afterwards, it spread to different countries all over the world. This disease is endemic in domestic and wild rabbits in Asia and Europe (Abrantes et al., 2012; Cooke et al., 2013). According to Cooke et al. (2013), Pedler et al. (2016) and Toh et al. (2022), the RHDV character is known for causing acute infections that can spread easily. The infection of RHDV has also reported first time in Singapore in 2020 where rabbits experienced clinical symptoms such as hyporexia, lethargy, pyrexia, and tachypnea (Toh et al., 2022).

The suspicions of RHD in Indonesia were confirmed in the Philippines in 2021. Rabbits that had been exported from Indonesia, their sera tested positive for RHD, but no symptoms of the disease were observed (unpublished data). Capucci reported the discovery of RHDV (also known as rabbit calicivirus (RCV) in a rabbit that showed no clinical symptoms of the disease in 1996 (Capucci et al., 1996). This finding revealed the presence of RHDV in apparently healthy rabbits. Another study found the non-pathogenic RHDV strain persists in wild rabbit populations in Australia. Furthermore, it has been found to offer partial cross-protection against lethal RHDV infection, experimentally. The non-pathogenic strain is known as RCV-A1 (Strive et al., 2009, 2010). According to Le Gall Recule's research in 2011, a non-pathogenic rabbit *Lagovirus* (NP-LV strain 06-11, GenBank/EMBL accession number AM268419), that is related to RHDV was discovered in healthy domestic rabbits in Australia. Through phylogenetic analysis, it was found that this strain is distinct but closely related to the Ashington strain and RCV. Unlike other non-pathogenic *Lagoviruses*, this strain induces antibodies that do not offer protection against RHDV (Le Gall-Reculé et al., 2011).

The initial study that has been conducted on asymptomatic rabbits, showed positive antibody titres of RHDV in West Bandung Regency, West Java Province, Indonesia (Setyaningsih et al., 2022). Further study is needed to strengthen the previous finding in West Bandung area, West Java where it is one of the central breeding rabbit in Indonesia. Therefore, the study took place in Lembang Subdistrict, which according to Priyanti and Raharjo (2012) and Hakim et al. (2022), Lembang is a Subdistrict in West Bandung Regency, West Java Province as one from some provinces in Indonesia where rabbits can be easily found since it is a well-known area for tourism and ecologically suitable for rabbit farming. The Lembang Subdistrict boasts a varied topography, characterized by undulating ridges and level plains. This region is renowned for its invigorating atmosphere, with an average temperature of 20°C and a humidity index of 84.63%. Precipitation levels vary among villages, with annual accumulations ranging from 1500-2500 mm (Ardhitya, 2014; Jefri, 2018). The environmental conditions play a crucial role in rabbit farming, with temperature being a significant factor affecting both reproduction and growth performance. The suitability of the conditions, therefore, becomes a crucial consideration for successful rabbit production. The average temperature in West Bandung Regency, specifically Lembang Subdistrict are suitable for rabbit farming where the range of ideal temperature for rabbittries is 15-25°C (Bodnar and Makra, 2019).

Although the study of seroprevalence of RHDV in rabbits being done previously, however no available report been published yet until now which examined the presence of the virus agent caused asymptomatic RHD within rabbits and the distribution of RHDV seroconversion based on rabbits' profiles. This study can provide an initial insight of the presence of RHDV antigen using DAS ELISA technique also the distribution patterns of RHDV in rabbits through sample profiling from seroconverted rabbits based on their age, sex, and breeds in West Bandung Regency, West Java Province, Indonesia.

MATERIALS AND METHODS

Samples collection

Three European rabbits (import breed) selected from specific farm which showed the most numbers of seroconverted rabbits from previous study. Two rabbits have been tested positive for their RHDV antibody using indirect ELISA technique and showed no clinical symptoms of RHDV while one rabbit is used as negative control and has been tested negative for RHDV antibody. Faeces samples were collected from the invidual cage. Rabbits were euthanized and organs such as liver and intestines were collected immediately after the rabbit died. The collected organs are then stored in separate containers to prevent contamination (Capucci et al., 1996). Organs were stored at -80 °C.

Samples preparation

Liver, intestines, and faeces collected were prepared according to DAS ELISA manufacturer's protocol. Each liver, intestine, and feces sample were weighed at approximately 1 gram and then macerated. These samples were then placed in labeled 1.5 ml microtubes. Next, approximately 2 ml of diluent was added to each microtube, and the samples were homogenized. After a thorough homogenization, the homogenate was centrifuged at 1000 rpm. The resulting supernatant will be utilized as the assay sample for DAS ELISA.

Double antibody sandwich (DAS) Enzyme linked immunosorbent assay to determine rabbit haemorrhagic disease (RHD) virus antigen titer

Rabbit Haemorrhagic Disease (RHD) antigen titer was

tested using DAS ELISA method from the Ingezim® RHDV ELISA Kit (R.17.RHD.K2) (Inmunologia Y Genetica Aplicada, S.A.). The plates in this kit are coated with polyclonal antibodies that are designed to target RHDV. In case the samples contain the antigen, it will bind to the polyclonal antibodies that have been coated on the plate. Once a MAb-PO, specific to the VP60 protein of RHDV, is introduced, it will bind to the antigen that has previously bound to the antibodies on the plate. The reaction is detected by observing a colorimetric change that occurs after the substrate is added. Testing was performed according to manufacturer's protocol. Briefly, positive and negative control of 100 µl each were added to wells A1 and B1 respectively. A total of 100 µl of the prepared organ sample suspension was added into each well, then the microplate was closed and incubated for 60 minutes at 37°C. The plates were washed 4 (four) times using the available washing buffer with a volume of 300 µl/well/wash. The prepared conjugate solution of 100 µl was added to all wells. The second incubation was carried out for 60 minutes at room temperature 25°C. The plate was washed 4 (four) times and then 100 µl of substrate was added to each well. The microplate was incubated for 10 minutes at room temperature before adding 100 l stop solution to each well. The plate was read using an ELISA reader at a wavelength of 450 nm 5 minutes after adding the stop solution.

Data analysis

The test results of DAS ELISA to check antigen titer were valida ted according to manufacturer's protocol. This involved comparing the optical density (OD) values where OD_{450} positive control > 1 and OD_{450} negative control < cut-off value. The cut off value is 15% of the OD_{450} of the positive control, that is 0.15 x OD_{450}. Samples with an OD value higher than the cut off are said to be positive results and samples with an OD value lower than the cut off are declared negative for the RHD virus.

Antibody titers of Rabbit Haemorrhagic Disease (RHD) been tested using indirect Enzyme Linked Immunosorbent Assay (ELISA) method from Ingezim® RHDV ELISA KIT (R.17.RHD.K1) (Inmunologia Y Genetica Aplicada, S.A.). As many as 163 serum samples were tested and evaluated following the factory's protocol.

Data profiling

Rabbits' profile such as sex, breeds and age were taken from previous study including antibody titres of RHDV from each rabbit. Those antibody titres of RHDV obtained from asymptomatic rabbits distributed at 13 rabbit farms (rabbitries) in 7 villages: Lembang, Pagerwangi, Cikahuripan, Cikole, Sukajaya, Gudangkahuripan and Jambudipa, in West Bandung Regency, West Java Province, Indonesia. Each rabbit informations (age, sex and breed) being matched with the samples then used for samples profiling. The group of age divided into young rabbits group (<9 months) and adult rabbits group (≥9 months) based on Laber-Laird et al. (1996). The group of sex divided into female and male rabbits while group of breeds are consist of local and imported/exotic group. All serological data based on rabbits profiling (age, sex and breed) will be analised using chi square-yates correction method.

RESULTS AND DISCUSSION

All samples showed negative antigen titres of RHDV using DAS ELISA technique (positive cut off value >0.334). The average OD_{450} of samples was approximately 0.04. This value was found to be lower than the average OD_{450} of the negative and positive control which was 0.047 and 2.226, respectively.

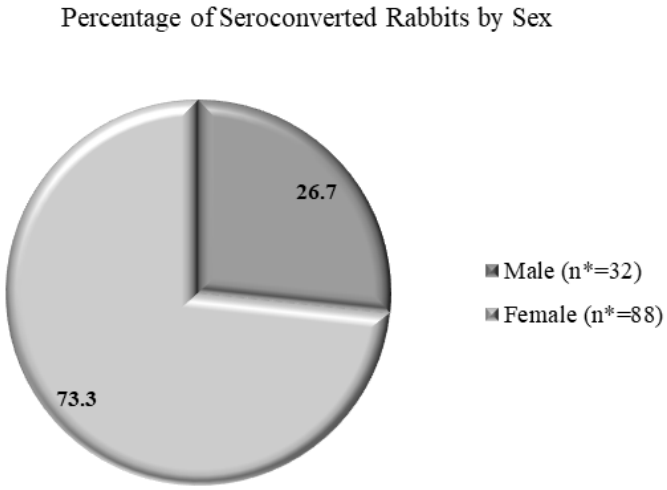

Figure 1: RHDV seroconversion percentage within female and male rabbits out of 120 seroconverted rabbits (n*=numbers of seroconverted).

Profiling data revealed that among the rabbits with positive antibody results, the majority were female rabbits (73.3% out of 120 seropositive samples) (Figure 1) and young rabbits (Figure 2) (72.5% out of 120 seropositive samples). The highest numbers of seropositive results were observed on rabbit farms located in Lembang, Sukajaya, Cikahuripan, Gudang Kahuripan, Cikole, Pagerwangi, and Jambudipa, respectively. It was discovered imported breeds (86.7%) and local breeds (13.3%) of rabbits showed seropositivity.

There is a strong suspicion that RHDV may be present among rabbits that have been exported from Indonesia and show no clinical signs, as they come from farms in the same areas where the study samples were taken and have RHDV-positive antibodies. It is currently unknown when and where RHDV was first introduced to Indonesia.

However, it is possible that the virus was brought into the country through rabbit imports. Indonesia has been importing rabbits from European and North American countries where RHDV is endemic, and there are no regulations in place requiring RHDV testing for imported rabbits. It is important to note that the rabbits included in this study have not been vaccinated in their country of origin.

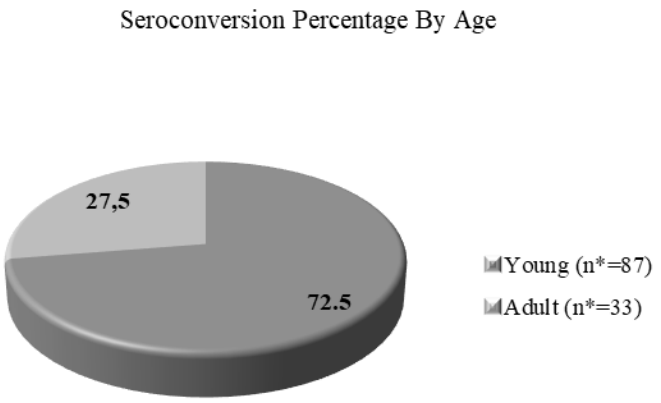

Figure 2: RHDV seroconversion percentage within group of age. It shows 72% (87 out of 120) seroconverted rabbits were young group rabbits (n*= numbers of seroconverted).

This study showed there were no antigen detected from seroconverted rabbits using DAS ELISA technique whether from liver, intestine or faeces samples. These results can be an indication of some possibilities such as the viral amount of samples are not sufficient to be detected using DAS ELISA, the affinity of Mab to bind with VP60 protein or surface epitope of the virus is weak (Collins et al., 1996), or RHDV in rabbits used in this study already removed by clearance mechanism of immune system. Collins et al. (1996) reported that the rabbits experimentally infected by classic RHDV (with clinical signs) failed to show positive results from faeces and urine samples. The study also showed the ability of urine to degrade viral antigen. Antigen detection using DAS ELISA is claimed to have more sensitivity than haemaglutination (HA) test for RHDV. The plate used for ELISA test have been coated with polyclonal antibody specific for RHDV while the antibody capture used is specific monoclonal antibody (Mab) for VP60 protein. According to Stanker and Hnasko (2015) the sandwich ELISA method necessitates the use of two distinct antibodies: A capture antibody and a detection antibody, both of which bind to a unique epitope on the target antigen. However, there is possibility that Mab used in sandwich ELISA method as a capture/tracer cannot bind properly with antigen surface or protein VP60 of the virus. Capucci et al. (1991) through his experimental study showed various reactivity between specific Mab, VP60 proteins and epitopes of the intact structure of the virus. His study also showed that ELISA can be resulted in false negative and hard to achieve a clear threshold of positivity, thus good ELISA results obtained from large amount of virions.

Although the seroconverted rabbits showed negative titre of RHDV antigen using DAS ELISA, there is possibility that RHDV can be detected using another diagnostic tests. For example, even HA test can be used for RHDV detection, but according to Chasey et al. (1995), HA tests may not always provide a conclusive diagnosis for RHDV outbreaks since some non-haemagglutinating isolates of RHDV, which are virtually identical to other strains, have been found in certain outbreaks. Therefore, it is necessary to use additional diagnostic methods to ensure accurate identification of the virus causing the outbreak. The same study also showed its concern to find suitable diagnostic method since they found seropositive rabbits without clinical signs of RHDV in UK. There is not much study can be found regarding the character of non pathogenic RHDV and the suitable diagnostic method to confirm its presence.

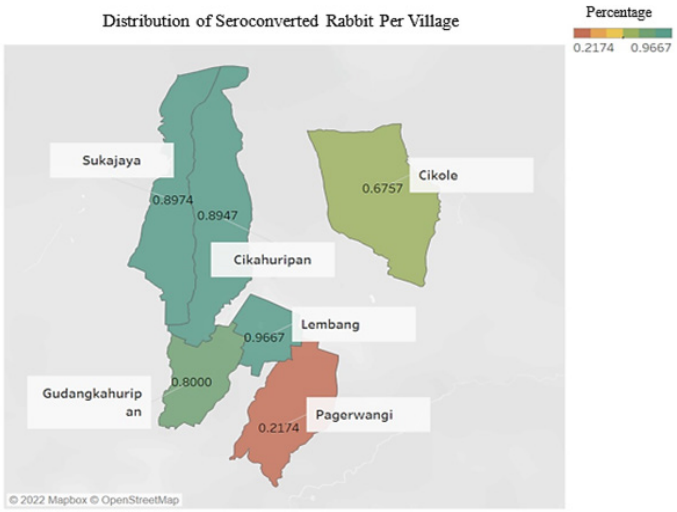

Figure 3: The percentage distribution of seroconverted rabbits infected by RHDV in 7 villages, West Bandung Area, West Java, Indonesia.

Data showed that RHDV seroconversion was found in rabbits without clinical signs on 13 farms in seven villages (Figure 3). Interestingly, area and rabbit farms were closer one to another. There are some factors which increases the risk of disease transmission such as low biosecurity practice or sanitation measures. This issue is particularly alarming due to the existence of non-pathogenic RHDV strains that do not display any discernible symptoms. Furthermore, these farms are either open or semi-open, allowing insects to easily enter. Cooke and Fenner (2002) reported some insects could potentially act as vectors to spread RHDV such as *Musca vestutissima* (bushflies) and *Calliphora* spp. (bowflies) (Cooke and Fenner, 2002). Their study also revealed that *Ochlerotatus* (*Aedes*) *postspiroculosus*

(mosquito) could carry RHDV. Through RT-PCR it can be revealed that the virus can stay up to 9 days in flies also the fresh faeces (fliesspots) is infective from flies that had been fed on RHDV infected rabbit livers (Asgari et al., 1988). A research conducted by Henning et al. (2005) has confirmed that RHDV can remain viable in animal tissues, such as rabbit carcasses, for a minimum of three months when exposed to the field. However, virus directly exposed to environmental conditions, such as dried excreted virus, can only survive for less than a month. This indicates that the persistence of RHDV in deceased animal tissues can serve as a reservoir for the virus, potentially resulting in new outbreaks of the disease even after a considerable delay (Henning et al., 2005). Effective biosecurity measures are critical in controlling and preventing the spread of RHD in the rabbit industry. These measures include surveillance, sanitation, disinfection, and quarantine, in addition to vaccination. Vigilant monitoring of viral evolution in the field is imperative for the prompt detection of new genetic and antigenic variants, which is crucial in determining the most appropriate course of action.

Capucci discovered that rabbits were experiencing seroconversion without showing any signs of disease related to the virus-like RHDV in 1995. The following year, in 1996, Capucci reported the presence of a non-pathogenic RHDV strain that had different characteristics from the RHDV in its classical form is known to cause significant mortality rates in rabbits. It is named as RCV to differentiate it from RHDV (Capucci et al., 1996, 1997). Research on non-pathogenic variants of RHDV in Australia was conducted by Strive et al. (2009, 2010), and France by Le Gall-Reculé et al. (2011). These variants, known as RCV-A1 and NP-LV strain 06-11 at the time, were studied extensively. Another variant of benign RHDV was reported by (Forrester et al., 2007) which called as Lambay strain. This strain is known to be silently presented among wild healthy rabbits in Lambay island. Kerr et al. (2009) conducted research that illuminated the correlation between RHDV and RCV. It is noteworthy to mention that this association was first established more than a century ago for the RHDV isolates that have been spreading globally since 1984. Thus, numerous studies have provided conclusive evidence that RHDV has been present among rabbit populations for a significant period, manifesting either as a persistent or latent infection, or in a subdued variant of the virus.

This study also delves into the susceptibility of rabbits to Rabbit Hemorrhagic Disease Virus (RHDV), taking into consideration their origin, age, and sex. Through our analysis, which is presented in Tables 1, 2, and 3, we have discovered the responses of imported and local rabbits to RHDV infection, age-related differences in susceptibility among young and adult rabbits, and the potential impact of rabbit sex on their vulnerability to this virus. These findings provide valuable insights into the epidemiological dynamics of RHDV and offer crucial information for the development of targeted management and prevention strategies, ultimately contributing to the overall health and well-being of rabbit populations.

Table 1: The seroconversion rate of RHDV in asymptomatic rabbits and *chi-square* based on breeds.

Breeds	Seroconversion (n)		Total	Chi-square count
	Positive	Negative		
Imported/Exotic	97	32	129	0,62
Local	23	11	34	
Total	120	43	163	

*The *chi-square* is counted using 2 x 2 yates correction method with α=0,05 and *chi-square* table 3.841.

Table 2: The seroconversion rate of RHDV in asymptomatic rabbits and *chi-square* based on age.

Age*	Seroconversion (n)		Total	Chi-square count
	Positive	Negative		
Young	87	37	124	10,19
Adult	33	6	39	
Total	120	43	163	

*The chi-square is counted using 2x2 yates correction method with α=0,05 and *chi-square* table 3.841.

Table 3: The seroconversion rate of RHDV in asymptomatic rabbits and *chi-square* based on sex.

Sex*	Seroconversion (n)		Total	Chi-square count
	Positive	Negative		
Male (n*=56)	32	24	56	34,26
Female (n*=107)	88	19	107	
Total	120	43	163	

*The *chi-square* is counted using 2x2 yates correction method with α=0,05 and *chi-square* table 3.841

At this study, group of breeds are divided into imported/exotic breeds and local breeds from the same species of *Oryctolagus cuniculus*, *Oryctolagus* genus. The seroconversion are shown by both local breeds and imported/exotic breeds of rabbits (New Zealand, Rex, Satin, Flemish Giant, Lop, Angora). Indonesian rabbits which later called as local breeds are actually *Oryctolagus cuniculus* which also called European rabbit that mixed with other breeds from the same species until its original breed is difficult to identify. The existence of European rabbit was first introduced by the Dutch to Indonesia, particularly Java, in 1835 for decorative reasons (Hustamin, 2008). Valentin et al. (2019), in his study wrote that, *Oryctolagus cuniculus*, commonly referred to as the European rabbit, is the sole representative of the *Oryctolagus* genus. This species is comprised of six distinct subspecies and is considered

the progenitor of all domesticated rabbit breeds. The European rabbit has demonstrated remarkable adaptability, successfully colonizing diverse ecosystems across the globe. Some of well-known breeds of European rabbit are New Zealand, Rex, Satin, Flemish Giant, English Lop, French Lop, Holland Lop, Dwarf Hotot, Blanc de Hotot, English Angora (Naff and Craig, 2012). According to WOAH (2023), the European or domestic rabbit (*Oryctolagus cuniculus*) is the only susceptible host species for RHDV and RHDVa (WOAH, 2023). This study found all breeds are susceptible to RHDV infection since seroconversion found in both local and imported/exotic breeds which is linear with WOAH (2023). The *chi-square* count (0,62) is smaller than *chi-square* table (α=0,05; 3.841) (Table 1), it indicates whether imported/exotic group or local group, both can show seroconversion because those rabbits are from the same species. The first case of RHD in 1984 also found in European rabbits, Angora breed, which imported from Germany to China (Liu et al., 1984). In a recent study by Elfekih et al. (2021), the susceptibility and resistance mechanisms of the European rabbit to RHDV were investigated. The study revealed that two genes, GYLTL1B and EXTL1, contribute to the resistance of European rabbits to RHDV in Australia. Similarly, Marques et al. (2012) found that IL-6 in European rabbits plays a significant role in their immune response against rabbit haemorrhagic disease virus (RHDV). Furthermore, Perkins et al. (2000) identified a specific mutation in the stop codon of the European rabbit that results in a protein with an additional 27 amino acids. These findings provide valuable insights into the genetic and immunological factors contributing to European rabbits' susceptibility to RHDV infection.

According to the data presented in Table 2, a total of 82 out of 124 young rabbits included in this study have exhibited seroconversion. The results of the *chi-square* analysis indicate a linear relationship between the age of rabbits and their seroconversion rate. Specifically, the calculated chi-square (34.26) exceeds the table *chi-square* (α=0.05; 3.841), indicating that age is a significant factor in determining susceptibility or resistance to RHDV infection in rabbits. Studies have indicated that young rabbits naturally exhibit a higher degree of susceptibility to RHDV exposure compared to their adult counterparts. Young rabbits are naturally resistant to RHDV infection due to the sustained proliferation of local and systemic B and T cells, resulting in a high seroconversion, in contrast to adults (Marques et al., 2012). This suggests that the high seroconversion observed in young rabbits may be attributed to the increased activity of their immune system. There are number of genes related to virally mediated immunomodulation being upregulated and showed a higher rate in young rabbits infected by RHDV compare to adults (Neave et al., 2018). The reasons why young rabbits showed higher percentage of seroconversion also related to the weaning period. Research about non-pathogenic RHDV conducted by Capucci et al. (1997) showed that young rabbits got passive immunity from their mother with high seroconversion (Capucci et al., 1997). Seroconversion is also believed to be caused by the virus circulating between the weaned rabbits and their mothers. The seronegative young rabbits could contract the infection from virus in the cage or litters and spread it to the environment then it is becoming a vicious cycle. The calicivirus infection in young rabbits triggered their immune response to produce specific antibodies against calicivirus. It will prevent RHDV infection when they become adults but at the same time young rabbits could be a major source of transmission of the virus since they may act as long-term carriers for the virus (Ferreira et al., 2004, 2008). Matthaei et al. (2014), through his study, showed that RHDV replicated and shed in young rabbits before they seroconverted compared to adults, playing an important role in virus transmission (Matthaei et al., (2014).

The study has revealed a higher proportion of female rabbits compared to males. This is because female rabbits are primarily utilized for breeding and fulfilling the market demand for juveniles. Interestingly, *chi-square* analysis to see if the sex of rabbits has a correlation with seroconversion of RHDV showed a significant result (Table 3). It means the sex of rabbits may play a role in the immune response against RHDV. In terms of antibody responses, it was observed that the collective strength of female rabbits was greater than that of males (Wang et al., 2010). The female reproductive tract is a component of the common immune response, particularly the mucosal immune system (McGhee et al., 1999). The secretory and serum derived-humoral immune response is detected in the genital tract after intranasal immunization, with a recombinant adenovirus expressing HSV-1 glycoprotein B. The immunization triggered a mucosal immune response that produce specific Ig G and Ig A (Gallichan and Rosenthal, 1995). Gallichan and Rosenthal (1996) also showed oestrus cycle is affecting the level of specific antibodies in the genital tract such as progesterone which affects the immune response related to the Ig G-Ig A ratios (Gallichan and Rosenthal, 1996). An experimental study by McAnulty and Molton (1978) showed that Ig G could be found in the serum and reproductive tract along with Ig A resulted from local immunization in the reproductive tract using horseradish-peroxidase (HRP) as an antigen while systemic immunization intramuscular only produced Ig G circulating in the body without the presence of Ig A (McAnulty and Molton, 1978). Furthermore, factors affecting immune response in female rabbit compared to male rabbits infected by RHDV need further study.

CONCLUSIONS AND RECOMMENDATIONS

There is no antigen could be detected in all samples on this study using DAS ELISA technique while seroconversion of asymptomatic rabbits exposed by non-pathogenic RHDV is affected by the rabbits' sex and age where the group of rabbits' breed has no significant correlation with RHDV seroconversion. This study also showed that seroconversion has spread within rabbit farms and all seven villages in West Java, Indonesia.

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NOVELTY STATEMENT

Our investigation in the West Bandung District of West Java Province in Indonesia has revealed an unexpected discovery. Despite seroconversion, commercial DAS ELISA kits were unable to detect RHDV antigen in asymptomatic rabbits. These finding challenges conventional thinking and demonstrates the complex dynamics of RHDV transmission in the area. Our research also uncovered intriguing patterns in seroconversion based on age and sex, which require further exploration. Furthermore, our findings suggest that RHDV spreads universally among asymptomatic rabbits regardless of their breeds in West Bandung Regency villages, Indonesia.

AUTHOR'S CONTRIBUTION

SM, RS, IWT, SS, EH: Equal authors, conducted the research, conceptualized the study, data analysis and finalized the manuscript. All authors have read, reviewed and approved the final content of the manuscript and agree to the conditions outline in the copyright assignment form.

Ethical approval and informed consent

The study was conducted according to the princi ples of the animal welfare. The protocol was approved by the animal ethics committee, School of Veterinary Medicine and Biomedical Science, IPB University. The ethical approval number is: 048/KEH/SKE/XI/2022.

Conflict of interest

The authors have declared no conflict of interest.

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