



SEROSURVEILLANCE ON JAPANESE ENCEPHALITIS VIRUS IN CHICKENS COLLECTED FROM TWO DIFFERENT GEOGRAPHICAL AREAS IN BALI, INDONESIA

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Summary

Kardena, I. M., A. A. A. M. Adi, N. M. Astawa, I. N. Suarsana & M. Bruce, 2021. Serosurveillance on Japanese encephalitis virus in chickens collected from two different geographical areas in Bali, Indonesia. *Bulg. J. Vet. Med.* (online first).

This study was performed to assess the seroprevalence against Japanese encephalitis virus (JEV) in chickens collected from an urban area of Denpasar and a rural area of Karangasem in Bali. A total of 142 domestic chickens were sampled and then tested using enzyme linked immunosorbent assay (ELISA) to detect the antibodies. The seroprevalence was calculated in each of the clustered areas and the seropositivity was associated with age and sex of the sampled chickens. The results showed that chickens collected in Denpasar had 97.10% (n=70, 95% CI: 90.88–99.52) seroprevalence against JEV, whereas the chickens collected from Karangasem Regency had a seroprevalence of 93.05% (n=72, 95% CI: 85.28–97.41). In association with area collection, age and sex, chickens collected from Denpasar were 2.5 times (OR: 2.5, 95% CI: 0.48–13.53, P>0.05) more likely to be seropositive containing the antibodies against JEV compared to the chickens from Karangasem Regency. Meanwhile, chickens of age equal to or more than six months were 6.2 times (OR: 6.2, 95% CI: 1.15–33.07, P<0.05) more likely to be seropositive compared to the chickens of age less than six months. The male chickens were 2.4 times (OR: 2.4, 95% CI: 0.45–12.76, P>0.05) more likely to be seropositive compared to females. No significant difference was observed for the seroprevalence in the chickens collected in both areas even though it was found to be significantly associated with the chickens' age.

Key words: chickens, ELISA, Japanese encephalitis, seroprevalence, survey

INTRODUCTION

Japanese encephalitis (JE) is a zoonotic viral disease caused by Japanese encephalitis virus (JEV). It is a member of *Flaviviridae*, in the genus of *Flavivirus*

(Mansfield *et al.*, 2017). The virus infection may result in fatal encephalitis in both infected animals and humans. The distribution of the virus infection is

through the mosquito vector, mainly by *Culex spp.*, especially *Culex tritaeniorhynchus* (Bae *et al.*, 2018). The viral transmission tends to circulate from pigs and wading birds as the amplifying hosts and the main reservoirs to other hosts through the vector bites (Cappelle *et al.*, 2016). Domestic birds like chickens and ducks can be exposed to the virus and are suspected to have a role in the disease occurrence (Cleton *et al.*, 2014; Lord *et al.*, 2015). Due to a wide range of animals that can be infected, the disease may cause a significant economic impact on both humans and livestock sectors (Tarantola *et al.*, 2014).

Japanese encephalitis is previously known to be a rural disease. More agricultural activities are found in the rural areas, especially in East and Southeast Asian countries where the JE is mainly endemic (Adi *et al.*, 2016; Cappelle *et al.*, 2016). However, recently the cases of JE have also been detected in the urban and peri-urban areas. This means that the distribution of the disease is getting wider geographically although the pig farms and rice paddy fields as the risk factors of the disease occurrence (Liu *et al.*, 2010; Vallée *et al.*, 2009) are less likely to be found in those areas.

The evidence of JE related cases in urban areas has reportedly increased. From the veterinary perspective, this might mainly be contributed by the bird hosts instead of pigs. Migratory and domestic birds may transmit the JEV through mosquito vector in the area where the population of pigs is sparse (Lord *et al.*, 2015). The period of viraemia in the infected birds, including young ducks and chickens that are reported to have the JEV titre, may sufficiently provide a chance for the mosquito vector to feed on them and spread the virus to other susceptible hosts

(Bhattacharya & Basu, 2014; Cleton *et al.*, 2014; Ladreyt *et al.*, 2020). This transmission, however, may contribute to risk of exposure and transmission of the virus into other hosts, including humans.

Surveys on antibodies against JEV detected in chickens have been globally performed. In Malaysia, along with other animals, chickens and birds among other suspected animals were surveyed for JEV antibodies detection collected in the high risk disease areas (Kumar *et al.*, 2018). Similarly, in Cambodia, the antibodies were also detected in the chicken samples collected in some urban and rural areas (Auerswald *et al.*, 2020). Meanwhile, in urban areas of Singapore, recent surveys found that the seroconversion of the JEV antibodies was detected in sentinel chicken samples (Yap *et al.*, 2019).

Indonesia is a Southeast Asian country endemically infected with JEV. In this country, 29 out of 33 provinces were reported to have cases of JE (Garjito *et al.*, 2018). Bali is a province in Indonesia nationally reported to have the highest JE cases in humans (Ambarawati *et al.*, 2020). However, the cases of JE in humans were not only found in rural areas, but they were also recorded as occurring in urban areas. Meanwhile, although the island of Bali has high density of pig population (Yamanaka *et al.*, 2010), the population in urban areas in Bali is much less than in the rural ones. However, chickens are the other domestic animals that tend to be reared by the local residents, both in rural and urban areas of Bali. As chickens may have a role in the disease transmission and they tend to be close to humans, a survey on JEV antibodies in chickens in those areas of Bali was performed to assess the JEV activity and identify the seroprevalence against JEV, in association with age and sex of the collected chickens.

MATERIALS AND METHODS

Sampling chickens

The study areas used in this study were Denpasar city and Karangasem Regency. Denpasar city is the main city on the island of Bali, Indonesia, while Karangasem Regency is a rural area on the island, located around 80 km removed from the city (Fig. 1).

Lists of the chicken farmers' names were gathered from the local animal health authorised staff. The farmers were randomly chosen, and their chickens were sampled prior to the farmers' approval. Three to five domestic chickens from each randomly selected farmer were bled to collect their sera. The fieldwork was conducted from February to March 2020.

Informed consent for the farmers

Informed consent was prepared and used to get the farmers' approvals. The chicken farmers or owners were informed about the aims of the survey and methods being used in collecting and bleeding their ani-

mals. The informed consent was delivered orally. Once the approvals were obtained, bleeding their animals was performed to obtain the sera.

Serum collection

The sampled chickens were bled 1–2 mL from brachial vein using 1 to 3 mL spites. The collected blood was clotted until the serum released. Each of the collected sera was centrifuged at 4000 rpm for 10 minutes before being stored in a -20°C freezer. A day before the serological test was performed, all of the sample sera were stored in a 4°C refrigerator. In total, 142 sera of domestic chickens (*Gallus domesticus*) were collected and used as the samples in this study. As many as 70 chickens were sampled in the urban area, Denpasar city, while in the rural area, Karangasem Regency, 72 chicken samples were collected.

The indirect ELISA test

A concentration of 0.5 ng/mL of the JEV antigen was made by mixing 7.5 μL JEV



Fig. 1. Map of Denpasar city and Karangasem Regency where the chicken samples were collected from.

(recombinant protein, Creative Diagnostics NY 11967, USA) and 15 mL carbonate bicarbonate coating buffer (0.1M carbonate buffer, pH 9.6). Coating was performed by filling 75 µL of the antigen dilution into each well of the 96 well microplates. The plates were sealed and incubated for 16 hours at a temperature of 4 °C. Then, the JEV coated microplates were washed three times by using Tween washing solution 0.01%. Blocking was conducted by adding 200 µL of 3% non-fat skim milk (Oxoid product) blocking buffer in each well. Then, the plates were covered with adhesive plastic and incubated for an hour in an incubator at 37°C. Next, the plates were washed three times by using the washing buffer. A 50 µL of 3% skim milk and 50 µL primary antibody in each well were added. Next, the plates were again covered with adhesive plastics and incubated for one hour in the incubator at 37°C. The plates were then washed three times with the washing buffer. Secondary antibody conjugate was added at 100 µL. Then the plates were again covered with adhesive plastics and incubated for one hour at 37 °C. The plates were then washed three times with the washing solution. A 50 µL substrate solution (tetramethylbenzidine/TMB peroxidase substrate A and peroxidase substrate B were mixed) was put into each well. Finally, after 15–20 minutes, a 50 µL stop solution (phosphoric acid) was added to the wells before the optical density (OD) value absorbance at 450 nm was recorded on the ELISA plate reader (ELx 800-Biokit). Cut off titre value of the ELISA test was determined by calculating the mean of all negative controls plus six times standard deviation (SD) of the negative controls. A sample serum was assigned to be positive for containing the antibodies against JEV when it had the OD value greater than the

cut off, while the serum sample that had the same and lower than the mean value of the negative controls was assigned to be negative.

Animal ethics

This protocol research has been approved by the Ethics Commission for the Use of Animals in Research and Education, Faculty of Veterinary Medicine, Udayana University with certificate reference number 14/UN14.2.9/PD/2020.

Statistical analysis

The seroprevalence was calculated by dividing the positive serum samples containing the antibodies with the total sample chicken sera tested adapted from Kumar *et al.* (2018). The seroprevalence was analysed in each of the study areas before being compared. Then, the seroprevalence was associated with age and sex of the chicken samples collected. Data of the prevalence, odd ratio (OR) with 95% confidence interval (CI), and a Mid P Exact significant level (two tailed P value) of 0.05 were analysed by using an open-source epidemiologic statistics for public health (OpenEpi version 3.01).

RESULTS

Chicken samples collected in Denpasar were 97.10% (n=70, 95% CI: 90.88–99.52) found to be seropositive containing the antibodies against Japanese encephalitis. However, the seroprevalence against JEV in chickens collected in Karangasem Regency was slightly lower at 93.05% (n=72, 95% CI: 85.28–97.41) (Table 1).

The collected chickens in Denpasar and Karangasem were then grouped based on their age, i.e., chicken samples less than six months of age and another group of chickens equal to or more than six

Table 1. Seroprevalence against Japanese encephalitis virus in chickens collected in Denpasar and Karangasem

Area	Sample number	Seropositive	95% CI
Denpasar	70	97.10%	90.88–99.52%
Karangasem	72	93.05%	85.28–97.41%
Total	142	95.07%	90.49–97.82%

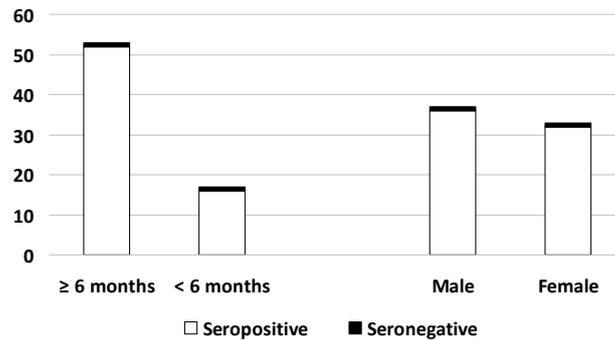


Fig. 2. Seroprevalence against JEV in chickens collected in Denpasar city associated with age and sex.

months of age, and sex. The chickens collected in Denpasar that were equal to or more than six months of age had a seroprevalence against JEV of 98.1% (n=53, 95% CI: 91.05–99.9), while the chickens that were less than six months had seroprevalence at 94.1% (n=17, 95% CI: 74.25–99.71). Similarly, 97.3% of male chickens collected in Denpasar were seropositive (n=37, 95% CI: 87.39–99.87) that was almost the same as 97% (n=33, 95% CI: 85.95–99.85) in the female chickens (Fig. 2).

Collected chickens in Karangasem that were equal to or more than six months of age had 97.8% (n=45, 95% CI: 89.53–99.89) antibodies against JEV, whereas the chickens that were less than six months had a slightly lower seroprevalence of 85.2% (n=27, 95% CI: 68.03–95.1). Based on sex, male chickens had a seroprevalence of 97% (n=31, 95% CI:

85.1–99.84), while the females: 90.2% (n=41, 95% CI: 78.12–96.82) (Fig. 3).

In comparison, chickens collected in Denpasar were 2.5 times (OR: 2.5, 95% CI: 0.48–13.53, P>0.05) more likely to be seropositive, containing antibodies against JEV, compared to chickens that collected in Karangasem Regency. No significant difference was observed in the prevalence of the antibodies against JEV between chickens sampled in Denpasar compared to the chickens sampled in Karangasem (P>0.05).

When all of the collected chickens from both Denpasar and Karangasem were gathered and grouped, the chickens aged equal to or more than six months were 6.2 times (OR: 6.2, 95% CI: 1.15–3.07, P<0.05) more likely to be seropositive containing the JEV antibodies compared to the chickens that aged less than six months. Meanwhile, in the

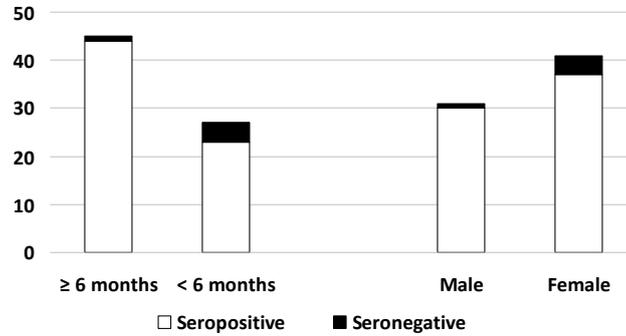


Fig. 3. Seroprevalence against JEV in chickens collected in Karangasem Regency associated with age and sex.

Table 2. Seroprevalence against JEV in the total collected chickens in association with the sampling area, age, and sex

Determinants	Sample number	Seropositive	OR	95% CI	Significance
<i>Area</i>					
Denpasar	70	97.10%	2.5	0.48–13.53	P>0.05
Karangasem	72	93.05%			
<i>Age</i>					
≥ 6 months	98	98%	6.2	1.15–33.07	P<0.05
< 6 months	44	88.60%			
<i>Sex</i>					
Male	68	97%	2.4	0.45–12.76	P>0.05
Female	74	93.20%			

group of male and female chickens, the male chickens were 2.4 times (OR: 2.4, 95% CI: 0.45–2.76, P>0.05) more likely to be seropositive compared to the female chickens. The comparison of association seroprevalence against JEV in the chickens collected within the areas, their age, and sex are illustrated in Table 2.

DISCUSSION

Chickens are reported to have high titre virus in viraemia when infected with the

JEV. It is suspected that the titre is high enough to be fed on by the mosquito vector before the virus is then dispersed to other susceptible hosts. Approximately more than 10^4 PFU/mL of virus were detected in one to three day old chicks in the viremia phase due to JEV infection and resulted in 10 to 100% transmission to the mosquito vector (Cleton *et al.*, 2014).

Formerly JE was known to be a rural disease in the area where the agricultural practices can be found more often. Wide distribution of irrigated rice paddy fields along with a massive household pig farm

industry are the specific practices found, and reported to be the risk factors of the disease occurrence (Liu *et al.*, 2010). In Bali, the cases of JE infection in children were associated with the close proximity to pigs or pig farms and rice paddy fields (Damayanti *et al.*, 2017).

A survey on antibody detection in the urban area and its surroundings is important to be conducted, as the incidence of JE cases in the area has been recently increasing. In Southeast Asian countries, JE cases have been increasingly found in urban areas and the vicinity of urban areas in Cambodia (Cappelle *et al.*, 2016) and Vietnam (Nguyen-Tien *et al.*, 2019). In Bali, Indonesia, a previous survey conducted in 2005/2006 found antibodies against JEV detected using ELISA test in 4.3% (n=23) of chicken samples collected in Denpasar, while in another rural area, Buleleng Regency, the antibodies were found in 58.3% (n=24) of the chicken samples. However, no chicken sample was collected in Karangasem Regency in the survey (Adi *et al.*, 2016). Although the survey method performed was not the same with this study, the comparison results show significant seropositive difference between the studies.

Surveys detecting the activity of JEV in urban areas are required as the disease is reported to be widely distributed to different geographical areas. Although the JEV distribution and circulation in city areas seem to be mainly contributed by wild and domestic birds (Hameed *et al.*, 2021), less likely by pigs and through the mosquito vectors, the JEV is also suspected to be transmitted to the chickens in the area. In urban areas of Singapore, where they decided to slaughter all of the pigs, sentinel chicken samples showed seroconversion of antibodies against JEV occurred. Wild and domestic birds were

suspected to be the contributors of JEV circulation in the area (Yap *et al.*, 2019).

Another reason that may contribute to the high prevalence of the antibodies detected in the chicken samples is that the domestic chickens in Bali tend to be kept longer, especially the roosters that the residents mainly use for the traditional games and ceremonies. In this study, the results of the chicken samples equal to or more than six months of age were more likely to be JEV seropositive compared to the female chicken samples. These results are in line with a survey detecting flavivirus antibodies, including JEV in ducks and chickens in Cambodia where the rate of the antibodies increased with the age of the birds. Birds that were less than three months were found to have the antibodies at 16.9% (n= 195), while 51% (n= 147) of the older birds had the antibodies (Auerswald *et al.*, 2020).

In addition, the chicken owners or farmers kept their domestic chickens free roaming so that the chickens may have been in contact with other viraemic JEV infected hosts through being bitten by infected mosquito vectors. Chickens having been in close contact with or reared close to pigs or other JEV reservoirs are at high risk of exposure or virus transmission. Even infected young chickens were found to have a viraemia phase that had enough viral titre to infect mosquito vectors when these vectors fed on them (Cleton *et al.*, 2014).

The results of high JEV antibodies detected in the collected samples indicated high circulation of the virus in the study area. These results also support that JE in Bali is hyperendemic (Kari *et al.*, 2006; Kardena *et al.*, 2021a) having already infected a wide range of the susceptible hosts around, including domestic chickens. In addition, JE is a multifactorial dis-

ease that involves multisectoral consideration (Impoinvil *et al.*, 2012), especially socio-environmental factors (Zhang *et al.*, 2018). Surveys of the JEV antibodies in chickens cannot determine the intensive exposure and transmission occurred. However, as chickens are animals that are also close to humans, these study results can be an indication of the JEV activity in the area, and more importantly, data to predict the risk of exposure into the surrounding public health.

Although the JEV antibodies were detected in chicken serums, indicating the animals were susceptible to the infection, the role of the domestic birds, including chickens in the disease transmission has not been well concluded. The involvement of chickens as the reservoir of the disease transmission needs to be studied further. Seroconversion studies using chicken sentinel settings in low pig population areas or in urban settings may help in understanding the role of the animals in the disease circulation, along with surveys on potential mosquito vector identification that may involve in the distribution of the virus in the study area.

The high results of the JEV antibodies seroprevalence may also be affected by the abundance and types of the mosquitoes around the hosts. Mosquitoes in Bali might be similar around its geographical area. *Culex* spp. are the main vector of the virus that can be found in this area (Kanamitsu *et al.*, 1979; Paramarta *et al.*, 2016). More specifically, the dominant mosquitoes in the city of Denpasar may be quite different compared to the mosquitoes in rural area in Karangasem Regency. In urban area *Culex quinquefasciatus* tend to be more frequent, while *Culex tritaeniorhynchus* is more common in rural area (Di Francesco *et al.*, 2018). Both of those mosquitoes along with other species

of *Culex* spp. and even with several other mosquito genus of *Anopheles* spp., *Aedes* spp., *Armigeres* spp., *Mansonia* spp. are reported to be the potential JEV vectors (Pearce *et al.*, 2018).

The role of the pigs as the JEV amplifying hosts is an important part in determining the source of the virus in the ecology. The density of pig population is high in Bali due to socio-cultural background of the Balinese. Along with the chickens, the mainly Hindu Balinese tend to use pigs or pork offerings in their traditional ceremonies (Kardena *et al.*, 2021b) They also commonly rear their pigs close to other animals, including chickens. In this condition, the mosquitoes may likely feed on them and the risk of the JEV transmission seems to be high as well.

It is acknowledged that no neutralization test was performed in this research. Neutralization tests are a gold standard diagnostic of JEV identification. The test is important to be performed for the samples collected in the study area where more than one flavivirus may circulate. The aim is to convince the diagnostics and avoid cross-reaction with other flavivirus antibodies. However, due to no Biosafety level 3 laboratory being available in the study area, no neutralization test was performed. Even so, the serological ELISA test performed is reported to have high sensitivity and specificity that are comparable with the plaque reduction neutralization test (PRNT), the gold standard test used for flavivirus diagnostic identification (Litzba *et al.*, 2010), including JEV. The ELISA is also one of the diagnostic tests recommended by OIE (OIE, 2019).

CONCLUSION

Prevalence of antibodies against Japanese encephalitis virus in chickens sampled in

Denpasar was 97.10%, while in sampled chickens of Karangasem was 93.05%. No significant difference was observed in the seroprevalence of the antibodies between the chickens collected in the urban area of Denpasar city and the rural area of Karangasem Regency in the island of Bali. However, a significant association between the seropositivity of the antibodies and age of the chickens was found, in which chickens that were equal to or greater than six months were more likely to be seropositive compared to the chickens that were less than six months of age.

ACKNOWLEDGEMENTS

This research was a part of the outputs of initiation collaboration between Udayana University, Bali, Indonesia and Murdoch University, Australia through Udayana International Research Collaboration funded by Institute for Research and Community Service, Udayana University with contract number B/20-71/UN14.4.A/PT.01.05/2020 and Murdoch International Post Graduate Scholarship, Murdoch University, Western Australia. The authors also thank Mr. Cary Chappell, B.A., M.A. Ed, an English Language Program Instructor at Kansas State University, America for reviewing the English grammar in this manuscript.

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Paper received 13.06.2021; accepted for publication 12.11.2021

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