Serological Investigation of Japanese Encephalitis Virus Infection in Commercially Reared Pigs, Southwestern Nigeria

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Summary
Japanese encephalitis virus (JEV) is a zoonotic arbovirus that causes abortion, stillbirth, and congenital defects in pigs, and epidemic encephalitis in humans. Currently, there is scarcity of information on JEV infection in pigs in Nigeria. Since the *Culex tritaeniorhynchus* vector of JEV is present in Nigeria and considering recent anecdotal reports of abortions and birth of weak piglets in some pig farms in southwestern Nigeria, there is a need for studies on the presence of the virus and its true burden among pig populations in the country. Serum samples (n=368) obtained from farm-reared pigs in four States of southwestern Nigeria were screened for JEV-specific IgG antibodies using a commercial ELISA kit. An overall JEV seropositivity of 35.1% (95% CI: 30.18 – 39.93%) was obtained, with detectable antibodies in pigs of all age groups, breeds, sex, and locations. Our results suggest natural exposure of these unvaccinated intensively reared pigs to JEV circulating silently in the swine population with significant association of the seropositivity with location (state/community in which the pig farms exist) and breed of the pigs studied. This first report of detection of anti-JEV antibodies in pigs in Nigeria indicates that JEV circulated among these pigs and underscores the need for active surveillance for JEV in humans, pigs, and mosquitoes to provide valuable epidemiological data for the design of effective control strategies against the virus, thus forestalling potential future outbreaks of the infection.

Introduction

Japanese encephalitis virus (JEV) is a member of the *Flaviviridae* family of viruses, which includes four genera: *Flavivirus, Pestivirus, Hepacivirus* and *Pegivirus* (MacLachlan and Dubovi, 2017). *Flaviviruses* are small, enveloped viruses, having a single-stranded positive-sense RNA genome of about 11,000 bases and virion diameter of 50 nm (Unni *et al.*, 2011). Three structural proteins (pre-membrane, core, and envelope), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) are encoded by the JEV genome. The seven NS proteins are critical for virus replication, with NS5 performing most of the RNA-dependent RNA polymerase activity. The E protein is the largest structural protein and is required for viral entry into host cells. It is also the principal target of humoral immunity (Chong *et al.*, 2019). There are five genotypes of JEV based on the nucleotide sequence of the E gene (Kumar, 2014).

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Culex (Cx.) tritaeniorhynchus mosquitoes are the major vectors of JEV, although the virus gene has been found in over 30 mosquito species belonging to the genera *Culex, Anopheles*, *Aedes, Mansonia,* and *Armigere* (Pearce *et al*., 2018). JEV has a wide range of vertebrate hosts throughout its natural life cycle with domestic pigs serving as its amplifying hosts while ardeid birds (herons and egrets) and wading birds are the main reservoirs (Mulvey *et al.,* 2021).

Apart from pigs and birds, bats are also susceptible to bites of the principal mosquito vectors of JEV. These hosts, with symptomatic or asymptomatic status, contribute to the proliferation and maintenance of JEV in circulation (Mackenzie *et al*., 2006; Yin *et al.,* 2010). In addition, many mosquito species are opportunistic vectors i.e., they operate as a connecting vector between the reservoir hosts and dead-end hosts, such as humans and horses (Mackenzie *et al.,* 2002). In pregnant pigs, JEV causes stillbirths, mummification, embryonic death, infertility, and abortions (Takashima *et al.,* 1988) which may result in significant economic losses to producers (Coker *et al*., 2011; Tarantola *et al*., 2014). After infection, these amplifying hosts are normally asymptomatic, albeit they develop high-titer viremia that allows the virus to spread to biting mosquitoes (Mansfield *et al.,* 2017).

Laboratory diagnosis of JEV infections in humans and pigs is usually achieved through virus isolation, serological testing, detection of the virus during the viremic phase or virus nucleic acid detection by reverse transcription-polymerase chain reaction (Jan *et al*., 2000; Do *et al*., 2015; Mansfield *et al*., 2017). Furthermore, Simon-Loriere *et al*. (2017) recently used high-throughput RNA sequencing to confirm coinfection of JEV and Yellow fever virus in a 19-year-old patient. In addition, the enzyme-linked immunosorbent assay (ELISA) is the most widely used tool for serodiagnosis of JEV infections worldwide. With this assay, previous workers detected moderate to high JEV seropositivity rates between 44.4% and 73.45% in pigs in Nepal, Cambodia, Malaysia, and Vietnam (Pant, 2006; Litzba *et al.,* 2010; Duong *et al.,* 2011; Kumar *et al.,* 2018; Lee *et al.,* 2019).

To our knowledge, there is no information on JEV infection in pigs raised in Nigeria although stillbirths, abortions, early embryonic death, and infertility have been observed in commercial piggeries in the southwestern region. Abortions and infertility in the swine industry have been attributed to other pathogens such as porcine reproductive and respiratory syndrome virus, porcine parvovirus, and classical swine fever virus (Aiki-Raji *et al.,* 2014, 2018). Considering the anecdotal reports of abortions and birth of weak piglets in some pig farms in southwestern Nigeria and the recent detection of anti-JEV IgG antibodies in febrile humans in the country (Udeze and Odebisi-Omokanye, 2022), this study was designed to investigate the presence of anti-JEV antibodies in commercially raised pigs in urban, peri-urban, and rural areas of southwestern Nigeria.

Materials and methods

Study Area and Animals

The pig serum samples used in this study were collected from Oyo State (Atiba, Akinyele, Oluyole, and Ibadan North local government areas), Ogun State (Obafemi-Owode local government area), Lagos State (Ikorodu local government area), and Osun State (Ife North local government area) (Figure 1). These States rank among the major pig-producing States in the country (Omodele et al., 2019). The pigs ranged in age from 0 to 36 months and belonged to three breeds including Large White (n=300), Duroc (n=37), and Landrace (n=31).

The sampled pigs were observed for presenting clinical symptoms while the prevailing environmental conditions on the farms visited were also recorded. Apparently healthy pigs and pigs with history of stillbirths, abortions, and infertility were sampled.

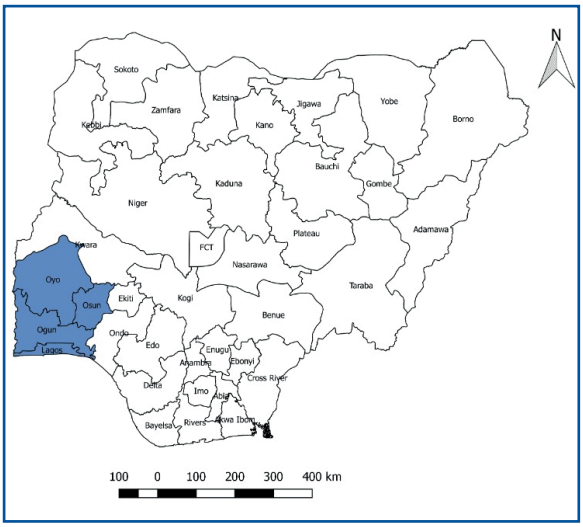

Figure 1. *Map of Nigeria Showing the Study Areas*

Sample Collection and Storage

Using sterile syringes and needles, about 3 ml of blood was aseptically collected from the anterior vena cava of each of 368 pigs in commercial piggeries located in Oyo (n=132), Ogun (n=92), Osun (n=85) and Lagos (n=59) States of southwestern Nigeria between November and December 2021.

The blood samples were dispensed into appropriately labelled anticoagulant-free sample tubes, screw-capped and transported on ice to the Virology laboratory, Department of Veterinary Microbiology, University of Ibadan, Nigeria. Upon arrival in the laboratory, the sera were separated from the clotted blood into labelled Eppendorf tubes and stored at -20°C until analyzed for anti-JEV antibodies.

Detection of anti-JEV Antibodies by Indirect ELISA

The sera were screened using an indirect ELISA kit (GreenSpring, Shenzhen, China) for the quantitative detection of anti-JEV IgG antibodies in swine serum. All the assay steps were carried out following the manufacturer's instructions and results were read using an ELISA microplate reader (BioTek Instruments Inc., Vermont, USA) at dual wavelength of 450 nm and 630 nm. The sensitivity and specificity of the ELISA were 94.0% and 96.0%, respectively.

Validation of ELISA Results

The results were judged by the Sample/Positive (S/P) ratio which was calculated using the formula:
S/P value = (Sample $OD_{450/630}$ - $\overline{NCX}$) / ($\overline{PCX}$ - $\overline{NCX}$) where:
$\overline{NCX}$ = Mean negative control $OD_{450/630}$ value
$\overline{PCX}$ = Mean positive control $OD_{450/630}$ value
For the assay to be valid, the mean optical density (OD) value of the positive control and negative control sera must be ≥ 0.6 and ≤ 0.1, respectively. Samples with S/P value ≥ 0.25 were considered positive while those with S/P values less than 0.25 were regarded as negative.

Data Analysis

Data obtained were presented with descriptive statistics (Table and Figure) with proportions and 95% confidence intervals (95% CI). The data obtained were analyzed with GraphPad prism version 9.3.1 (GraphPad software, San Diego, California) using binary logistic regression for analysis of association between proportions and the categorical variables (location, sex, breed, and age).
Odds ratio (OR) and 95% CI were estimated, and the level of statistical significance was set at ≤ 0.05.

Ethics Statement

The pigs used in this study were handled according to the guidelines provided by the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC/092-1121/18).

Results

The clinical symptoms observed among pigs on majority of the farms visited included anorexia, lethargy, and stunted growth, while some farmers reported they had experienced cases of infertility, frequent abortions, and variation in litter size.

The pig farms in Osun and Ogun States were observed to be close to streams, while the drainages around the pig pens were blocked with grasses and wastes generated from the farms that might encourage breeding/existence of mosquito vectors of JEV.

The mean OD values of the positive and negative control sera were 1.892 and 0.010, respectively.

Out of the 368 sera tested, 129 were positive giving an overall JEV seropositivity of 35.1% (95% CI: 30.18% – 39.93%) (Table 1).

Comparison of JEV seropositivity based on State of sample collection showed that pigs from Osun State had the highest seropositivity of 56.5% with odds ratio (OR) of 6.4 i.e., they had more than 6 times higher likelihood (p=0.0005) of seropositivity compared to pigs in Lagos State.

Similarly, pigs in Oyo State had more than 3 times higher likelihood (p=0.001) of seropositivity compared to those in Lagos State. However, pigs in Ogun and Lagos States were statistically comparable in seropositivity (OR=0.96, p=0.92).

Significant association was also observed between JEV seropositivity and the type of community in which the pigs were reared as those from farms located in urban communities had 5.6 times higher likelihood (p=0.0005) of seropositivity when compared with those in peri-urban communities.

Also, pigs in rural areas were more than twice likely to be seropositive compared to those in peri-urban areas (Table 1).

The breed of pigs also had significant association with JEV seropositivity as Large White pigs had more than three times (OR=3.1, p=0.014) higher possibility of seropositivity compared to the Duroc pigs.

However, although the Landrace breed had more than twice higher likelihood of seropositivity compared to the Duroc pigs, the difference was not statistically significant (p=0.126)

In addition, we observed that age (p=0.100) and sex (p=0.235) of the pigs were not significantly associated with JEV seropositivity (Table 1).

Nevertheless, of the 215 female pigs screened in this study, 55 (25.6%) had previous history of abortions while only 12 (21.8%) of these sows with previous abortions were seropositive for JEV.

Table I. *Seropositivity of JEV in pigs in southwestern Nigeria.*

Variable / Factor	No. tested	No. positive for anti-JEV antibodies (%)	95% CI of proportion	Odds ratio (95% CI)	p-value
Location (State)					
Oyo	132	56 (42.4)	33.99-50.86	3.6 (1.68-7.74)	0.001*
Lagos	59	10 (17.0)	7.38-26.52	1	
Osun	85	48 (56.5)	45.93-67.01	6.4 (2.85-14.20)	0.0005*
Ogun	92	15 (16.3)	8.76-23.85	0.96 (0.40-2.29)	0.92
Location (Community)					
Urban	59	36 (61.0)	48.57-73.46	5.6 (2.97-10.57)	0.0005*
Peri-Urban	174	38 (21.8)	15.70-27.98	1	
Rural	135	55 (40.7)	32.45-49.03	2.5 (1.5-4.05)	0.0005*
Sex					
Male	153	59 (38.6)	30.85-46.27	1.3 (0.84-2.00)	0.235
Female	215	70 (32.6)	26.29-38.82	1	
Breed					
Large White	300	113 (37.7)	32.18-43.15	3.1 (1.26-7.72)	0.014*
Duroc	37	6 (16.2)	4.34-28.09	1	
Landrace	31	10 (32.3)	15.8-48.71	2.5 (0.78-7.80)	0.126
Age (months)					
0-12	294	97 (33.0)	27.62-38.37	1	
> 12	74	32 (43.2)	31.96-54.53	1.55 (0.92-2.60)	0.100
Total	368	129 (35.1)	30.18 – 39.93		

** Significant association*
Odds ratio of 1 represents the reference group.

Discussion

Considering the scarcity of information on the status of JEV among pigs in Nigeria, this study was designed to investigate presence of the virus and its true burden among pig populations in the study area. To achieve this, detection of anti-JEV antibodies in pigs reared on farms in the study area was used as an indicator of previous exposure to the virus as the pigs had no history of JEV vaccination.

Japanese encephalitis is an emerging disease of animals and humans which is spread by mosquitoes to a range of susceptible wild and domestic vertebrate hosts (Mackenzie *et al.,* 2001). According to Daniels *et al.* (2002), endemic cycling of the causative JEV from mosquitoes to ardeid water birds creates a reservoir for spill-over to other species in periods of peak transmission, generally associated with warm-month high vector abundance. At such times, infection of pigs raised close to human habitations correlates with the occurrence of human disease since high viremic levels in pigs can promote infection of mosquito vectors that can readily transmit the virus to humans. To our knowledge, there has been no previous report of JEV infection in pigs in Nigeria. Cases of abortion and infertility in swine are often attributed to other causes such as porcine reproductive and respiratory syndrome virus, porcine parvovirus, and classical swine fever virus (Aiki-Raji *et al.,* 2014, 2018). Since the *Cx. tritaeniorhynchus* vector of JEV is present in Nigeria (Faizah *et al.,* 2020) and considering recent anecdotal reports of incidence of abortions and birth of weak piglets in some pig farms in southwestern Nigeria, we investigated possible circulation of the virus in commercial piggeries located in urban, peri-urban, and rural areas in four States of this region as a step towards evaluating the risk of virus transmission to humans, especially occupationally exposed persons and people residing near pig farms.

The detection of anti-JEV antibodies in the pigs tested in this study irrespective of farm location, sex, breed, and age group implicates JEV as a pathogen contributing to the cases of abortions, stillbirth, and infertility observed in pigs in the study area. Moreover, since vaccination against JEV is not practiced in Nigeria, the overall seropositivity of 35.1% obtained in this study indicates that commercially reared pigs in southwestern Nigeria have been naturally infected with the virus which seem to be circulating unnoticed in the pig population. Although this JEV seropositivity rate is comparable to the 44.4%, 48.1% and 49.0% obtained in Malaysia, Nepal, and Bali, Indonesia, respectively (Pant, 2006; Yamanaka

et al., 2010; Kumar *et al., 2018)*, it is considerably lower than the 65.7%, 74.7%, and 73.45% reported by other workers (Duong *et al.,* 2011; Conlan *et al.,* 2012; Lee *et al.,* 2019) in Cambodia, Laos, and Vietnam, which are southeast Asian countries known to be endemic for the disease. It is likely that migration of the natural JEV reservoir hosts i.e., ardeid wading birds such as egrets and herons from endemic countries, and the ongoing global climate change, which provides opportunity for increased distribution of the competent mosquito vectors (Soman *et al.,* 1977; Mulvey *et al.,* 2021) could have facilitated transmission of the virus to Nigeria.

Interestingly, the state and community (urban, peri-urban, and rural areas) in which the sampled pig farms were located correlated with JEV seropositivity. The detection of JEV-seropositive pigs in all four states suggests that the infection is widespread in southwestern Nigeria. Our results show strong correlation of JEV seropositivity to urban areas, although JE is historically considered a disease of rural areas. Urban settings have been shown to meet the two prerequisites necessary for spread of JEV, namely presence of competent *Cx. tritaeniorhynchus* vectors and the main amplifying host i.e., pigs (Do *et al.,* 1994; Lindahl *et al.,* 2012), while the number of vectors close to pigs has been shown to increase in urban areas (Lindahl *et al.,* 2012). Also, the highest JEV seropositivity obtained for pigs reared in urban areas despite their least sample size could be attributed to probable more intense activity of JEV-infected mosquitoes in these urban areas. Although pigs from both urban and rural areas had significantly higher seropositivity compared to those in the peri-urban areas, the possible reasons for this are not known. We advocate further studies to investigate and compare JEV mosquito vectors, their abundance and competence in the three communities.

Moreover, although the 38.6% JEV seropositivity rate obtained for boars in this study was higher than that of sows (32.6%), sex was not significantly correlated with serologic status (p=0.235). Similarly, despite the detection of higher JEV antibody prevalence in female pigs compared to the males, other workers (Thakur *et al.,* 2012; Kumar *et al.,* 2018) found no significant difference in seropositivity between gender. In addition, our results showed that adult pigs (i.e., pigs older than 12 months of age) were more predisposed to JEV infection than the younger ones, although the differences were not statistically significant. This is consistent with the findings of previous workers who also reported age-dependent increase in JEV seropositivity (Yamanaka *et al.,* 2010; Duong *et al.,* 2011; Lee *et al.,* 2019). The detection of anti-JEV antibodies in these adult pigs is an indication of natural infection of the animals rather than maternal immunity as maternal antibodies have been reported to protect piglets against JEV infection only for a period between 3-6 months (Scherer *et al.,* 1959; Nitatpattana *et al.,* 2011). The lack of association between JEV seropositivity and sex/age of pigs in this study could be due to the fact that JEV mosquito vectors did not discriminate between sex and age of the pigs when seeking hosts for blood meal.

Our observation that the State in which the pig farms were located was associated with JEV seropositivity as evidenced by significantly higher OR values for Osun (OR=6.4) and Oyo (OR=3.6) States is noteworthy. The closeness to a stream and presence of blocked drainages that might encourage breeding of mosquito vectors observed in the pig farm in Osun State might account for the significantly higher JEV seropositivity compared to that of Lagos State, while the highest sample size recorded for pigs in Oyo State might explain the significantly higher seropositivity obtained there. Also, we found that the breed of pigs was significantly associated with JEV antibody positivity as the Large White pigs had the highest seropositivity and were more than three times likely to be seropositive (p=0.014) than the Duroc breed. This observation could be because the Large White pigs had the largest sample size. Further studies to determine the host preference of JEV mosquito vectors among these pig breeds are needed.

Furthermore, the detection of relatively high overall JEV seropositivity (35.1%) in this study provides evidence for possible circulation of JEV in all settings (urban, peri-urban, and rural areas) in southwestern Nigeria and suggests a risk of human infection, especially of occupationally exposed persons such as pig farm attendants or owners, pig butchers and veterinarians in the study area. This becomes more germane considering previous reports that there is a correlation between the detection of JEV in pigs and incidence of encephalitis in humans (van den Hurk *et al.,* 2009; Ricklin *et al.,* 2016; Kumar *et al.,* 2020; Park *et al.,* 2022). Therefore, our findings underscore the need to screen humans, especially pig farmers/butchers, veterinarians, and people living in the vicinity of pig farms for possible exposure to JEV as they are at risk of contracting the virus from infected pigs via mosquito bites. Additionally, our results are consistent with the reports of previous workers (Lindahl *et al.,* 2013; Cappelle *et al.,* 2016) who also observed JEV circulation in pigs in urban and peri-urban environments and highlighted the importance of considering the risk of JEV transmission in these settings, and not only in rural areas as earlier reported (Mackenzie *et al.,* 2006; le Flohic *et al.,* 2013).

A major challenge to this study is the non-availability of cell culture facility and positive control JEV for performing plaque reduction neutralization

test which is the gold standard and more specific serological assay for diagnosis of JEV infections (Maeki *et al.,* 2019), since JEV is known to exhibit antigenic cross-reactivity with other flaviviruses such as West Nile virus, St. Louis encephalitis virus, Usutu virus and Murray Valley encephalitis virus (Calisher *et al.,* 1989; Yun and Lee, 2014). Another limitation of the study is the fact that sample collection was done over a limited period from November to December 2021 which covers only the dry season, thus excluding the rainy season during which JE transmission intensifies due to increased vector populations (WHO, 2019).

From the results of this study, it is evident that JEV had circulated among the sampled pigs prior to our investigation. Based on our findings and considering the zoonotic nature of the virus, we recommend active large-scale surveillance for JEV in the Nigerian swine population involving more States, a larger sample size, and sampling of mosquitoes and occupationally at-risk persons. The surveillance, which should span both rainy and dry seasons, should involve the use of serological methods that have been reported to reveal the presence of virus in the absence of overt disease (Yang *et al.,* 2007), and molecular screening for JEV nucleic acid. In addition, efforts should be directed at discouragement of pig rearing around human habitations, elimination of mosquito breeding sites around pig farms and residential areas, immunization of swine with JEV attenuated vaccine (Sasaki *et al.,* 1982), and creation of public awareness on JEV infection in Nigeria.

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