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Original Research

Surveillance of *Brucella suis* in Pigs from Selected Slaughterhouses in Luzon, Philippines Using Serological and Molecular Assays

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Abstract

Background: One of the most significant diseases for swine, including domestic pigs and wild boars, in terms of both public health and economics is brucellosis. This disease, which is brought on by the bacterium *Brucella suis* (*B. suis*), is regarded as a zoonotic disease that can spread from animals to humans and is a serious public health risk. The most recent report on the prevalence of swine brucellosis in the Philippines was published in the 1990s, thus there is neither updated nor recent data available to the public. Thus, the purpose of this investigation was to detect *B. suis* in pigs in selected provinces in Luzon, Philippines using qPCR and ELISA. **Methods:** To detect *B. suis* antigen and antibody, 115 blood samples of pigs from 15 slaughterhouses spread across 8 provinces were gathered and subjected to qPCR and ELISA. **Results:** *B. suis* DNA and anti-*Brucella* LPS antibodies were not detected in any of the 115 blood samples as demonstrated by qPCR and ELISA tests, respectively. **Conclusions:** In general, this study's findings offered updates on the *B. suis* infection status, indicating that pigs from the 8 provinces of Luzon, Philippines, did not have the *B. suis* chromosome 2 biovar 1 gene in their blood.

Keywords

ELISA, qPCR, swine brucellosis, *B. suis*

1. Introduction

Brucellosis is a significant bacterial zoonotic disease that affects not only a variety of livestock, including wild animals, sheep, goats, and camels but also humans [1]. The disease has been present in Egypt since 750 BC [2]. Bang's disease, infectious abortion, enzootic abortion, undulant fever, Malta fever, and Mediterranean fever are some other names for the disease [3].

B. suis causes brucellosis in pigs, a bacterial infection that results in persistent inflammation of the female and male reproductive systems as well as bacteremia. Currently, there are five biovars of *B. suis*, with biovars 1, 2, and 3 being in charge of brucellosis in pigs. Human sickness can be caused by biovars 1 and 3, although biovar 2 seems to be a rather uncommon cause of human disease [4]. Despite being categorized as zoonotic, biovar 4 isolates are exclusive to the subarctic, where they mostly infect reindeer and wild caribou (*Rangifer tarandus*). This biovar has also been observed in wolves (*Canis lupus*), moose (*Alces alces*), and arctic foxes (*Alopex lagopus*) in

subarctic regions [5]. Since the Biovar 5 strains have only been found in rats in the Soviet Union, their geographic spread is likewise severely restricted [6].

The prevalent clinical manifestations of swine brucellosis include reduced litter size, poor piglets born (potentially preterm), miscarriage, stillbirth, and other reproductive losses. Boars can occasionally get orchitis and epididymitis, which can affect fertility. Pigs that are not pregnant may not show any symptoms, but occasionally they get abscesses, become arthritic, and have posterior paralysis from spondylitis [7].

The disease can be transmitted from pigs to humans by direct exposure to live infected pigs or pig carcasses. Veterinarians, swine farm workers, slaughterhouse workers, butchers, and hunters who have frequent direct contact with live pigs, carcasses, aborted fetus, placenta, and pork products are at risk of contracting brucellosis [8-10]. Laboratory personnel who handle Brucella cultures and infected samples are also at risk [10]. Humans can also be infected by consuming inadequately cooked contaminated pork or pork products [8]. Despite the relatively high risk of transmission of the disease from infected pigs to humans, human-to-human transmission is rare and have only been implicated in exceptional cases involving blood transfusion, bone marrow transplant, sexual contact, nursing, and direct contact the patient or contaminated material during assisted childbirth [7].

Laboratory diagnosis of swine brucellosis includes serological testing and isolation from the clinical material. The diagnostic tests for swine brucellosis approved by WOAH are the Rose Bengal Plate Test (RBPT), Fluorescence Polarization Assay (FPA), Complement Fixation Test (CFT), and Enzyme-Linked Immunosorbent Assay (ELISA) [10]. Although the reliability of serological tests is good, it suffers from the problem of false positives that reduce their specificity. This is primarily due to the cross-reactivity of antibodies against *Yersinia enterolitica*, *Salmonella*, *Francisella,* and some other zoonotic pathogens [11].

B. suis affects pigs worldwide, although the disease has been either eliminated or nearly eliminated in some well-developed countries including the US, Canada, Australia, and some countries in in Europe [7,10]. The prevalence of swine brucellosis can be very high in South America and Southeast Asia, and the disease may be an unrecognized problem in some countries [10]. Swine brucellosis appears to be endemic in regions of Central and Southeast Asia, where there are high levels of pig production, sporadic outbreaks of *B. suis* (biovars 1 and 3) in pigs in the People's Republic of China with the greatest economic impact, and human infection with *B. suis* biovar 3 has also been reported [12, 13]. In Bangladesh, brucellosis in swine is also reported [14]. Epidemiological data on the occurrence of swine brucellosis in other parts of Southeast Asia are reported in Cambodia [15], Indonesia [16], Malaysia [17], Thailand [18], and Singapore [19].

In the Philippines, brucellosis in dogs [20] has been reported. In 1937, brucellosis in pigs was described and reported by isolation and identification of *B. suis* from pigs slaughtered at the Manila abattoir showing an incidence of 2.2% [21]. The study conducted by Tiglao (1990) [22] also found that the prevalence of *B. suis* in the Philippines was 17.62% which spread in 13 provinces of the Philippines. Since the last report on the prevalence and incidence of swine brucellosis in the Philippines was published in the 1990s, there was no recent data or updated information that was available to the public. Therefore, this study was conducted to identify *B. suis* in selected slaughterhouses in Luzon, Philippines and provide valuable information on the current incidence of swine brucellosis, in support to the surveillance efforts on zoonotic diseases contributing to the implementation of science-based control and prevention strategies against *Brucella* infections.

2. Materials and Methods

2.1 Research Design

The study used a cross-sectional study to extract data from the collected samples and information appropriately. The provinces and municipalities for sample collection as well as theClass A and AA municipal slaughterhouses, which cater to backyard farms only, were randomly selected for sampling. Blood samples were collected from pigs in selected slaughterhouses across the provinces in Luzon, Philippines, ranging from 11 to 18 samples per

province. A total of 115 pig blood samples were obtained in selected slaughterhouses in the 8 provinces—Cavite, Laguna, Batangas, Nueva Ecija, Camarines Sur, Benguet, Occidental Mindoro, and Pampanga (Table 1).

Table 1. Distribution of tested swine for swine brucellosis by provinces

PROVINCES	N	FREQUENCY (%)
Cavite	18	15.65
Laguna	13	11.30
Batangas	11	9.57
Nueva Ecija	15	13.04
Camarines Sur	14	12.17
Benguet	13	11.30
Occidental Mindoro	16	13.91
Pampanga	15	13.04
Total	115	100.00

ELISA and real-time PCR were used for the detection of the bacterium. The ELISA was used in the study since it has a maximum sensitivity of 97.2% and specificity of 98.3% [23], whereas the average efficiency of real-time PCR is 95% and 100% specificity [24].

2.2 Sample Collection and Preparation

Prior to sampling, the study was properly communicated with the Provincial Veterinary Offices (PVOs). The researchers sought coordination and obtained endorsement for access to the selected municipal slaughterhouses. Convenience sampling was used for the collection of the blood samples. The sampled pigs from the slaughterhouses were properly restrained to collect 4-5 mL of blood in the jugular vein. Then, sera were separated from the blood samples by centrifuging blood samples at 5000 rpm for 11 min at 8°C and were stored at -20°C for ELISA.

2.3 Serological Analysis

In the serological assay, the ID Screen® Brucellosis Serum Indirect Multi-species (ID. Vet, France) kit was used in duplicate serum samples. In the prepared 96-well plate, 190 µL of the dilution buffer, provided by the kit, was added, and then, each well was filled with 10 µL of the test serum, positive control, and negative control serum, respectively. The plate was covered and incubated at room temperature for 45 ± 4 min. After the incubation period, washing was done three consecutive times by adding 300 µL Wash Solution in the emptied wells. After, 100 µL conjugate was added to each well and then incubated at room temperature for 30 ± 3 min. Once done, a second washing was conducted three times. Incubation was done again after the addition of 100 µL substrate solution at room temperature for 15 ± 2 min. To stop the reaction after the incubation period, 100 µL stop solution was introduced to each well.

Using the ELISA reader (Thermo Fisher, USA), the absorbance at 450 nm was measured. If the ratio of the mean values of the positive and negative controls was larger than three, and the mean value of the absorbance of the positive control was greater than 0.350, the test was deemed valid.

2.4 DNA Extraction

DNA extraction was performed using GF-1 Tissue Blood Combi DNA Extraction Kit (Vivantis Technologies Sdn. Bhd., Malaysia) following the manufacturer's instructions. Briefly, approximately 200 µL of samples were placed in a 1.5 mL microcentrifuge tube, 200 µL of Buffer BB and 20 µL of Proteinase K were added, mixed, and incubated at 65°C for 10 min. Then 200 µL of absolute ethanol was added to the mixture, mixed immediately, and transferred to a column. Then centrifuged and discarded flow through. The samples were washed thrice with 500 µL wash buffer, centrifuged, and discarded flow through. The DNA was eluted by centrifugation at 5,000 x g for 1 min. Finally, the extracted DNA was stored at -20°C before further analysis.

2.5 Real-time PCR Assay

Using the GoTaq® Probe qPCR Master Mix (Promega [A5] Corp, USA) and the TOptical Gradient 96 Real-time PCR Thermal Cycler product from Biometra Analytik Jena Company (Biometra GmbH, Germany), all of the extracted DNA samples were tested in duplicate via real- time PCR. Specific primers of oligonucleotides namely, *B. suis* forward 5′-GCC AAA TAT CCATGC GGG AAG-3′ and *B. suis* rev 5′-TGG GCA TTC TCT ACG GTG TG-3′ were used targeting a 106 bp fragment of the

BS1330_II0657 locus encoded on chromosome 2 of *B. suis*. An additional FAM-labelled hydrolysis probe 6-carboxyfluorescein (FAM)-TTG CGC TTT TGT GAT CTT TGC GCT TTA TGG-BHQ1 was used alongside the primers for real-time PCR detection assay [24]. With minor modifications, real-time PCR was carried out using Hansel et al.'s [24] procedures: 95°C for 10 min and 50 cycles of 95°C for 15 sec and 60°C for 1 min. To guarantee the validity of the test results, all samples were examined in conjunction with the positive and negative controls. qPCRsoft
3.2 Software was used to automatically set the threshold and Ct values in real-time PCR machine.

2.6 Data Analysis

The descriptive statistic and incidence were calculated in Microsoft Office Excel 2019. Incidence of *B. suis* infection (%) were computed using the formula:

$$\text{Incidence of } B.\ suis \text{ infection (\%)} = \frac{\text{Number of positive samples}}{\text{Total number of samples}} \times 100$$

3. Results

A total of 115 blood samples collected from the slaughtered pigs in various Class A and AA municipal slaughterhouses, which cater to backyard farms only, in 8 provinces in the Philippines were tested using ELISA and real- time PCR assay to test for the presence of *B. suis DNA and anti-B. suis antibodies*. The results showed that all samples were negative for *B. suis* (Figure 1). Table 2 presents the incidence of swine brucellosis according to provinces and slaughterhouses using ELISA and real-time PCR assay. As shown in Table 2, all samples tested were negative for *B. suis* by both ELISA and real-time PCR assay. The results demonstrated that there were no *B. suis* antibodies and *B. suis* chromosome 2 biovar 1 gene in each tested sample. Hence, the incidence of *B. suis* infection in all samples was zero.

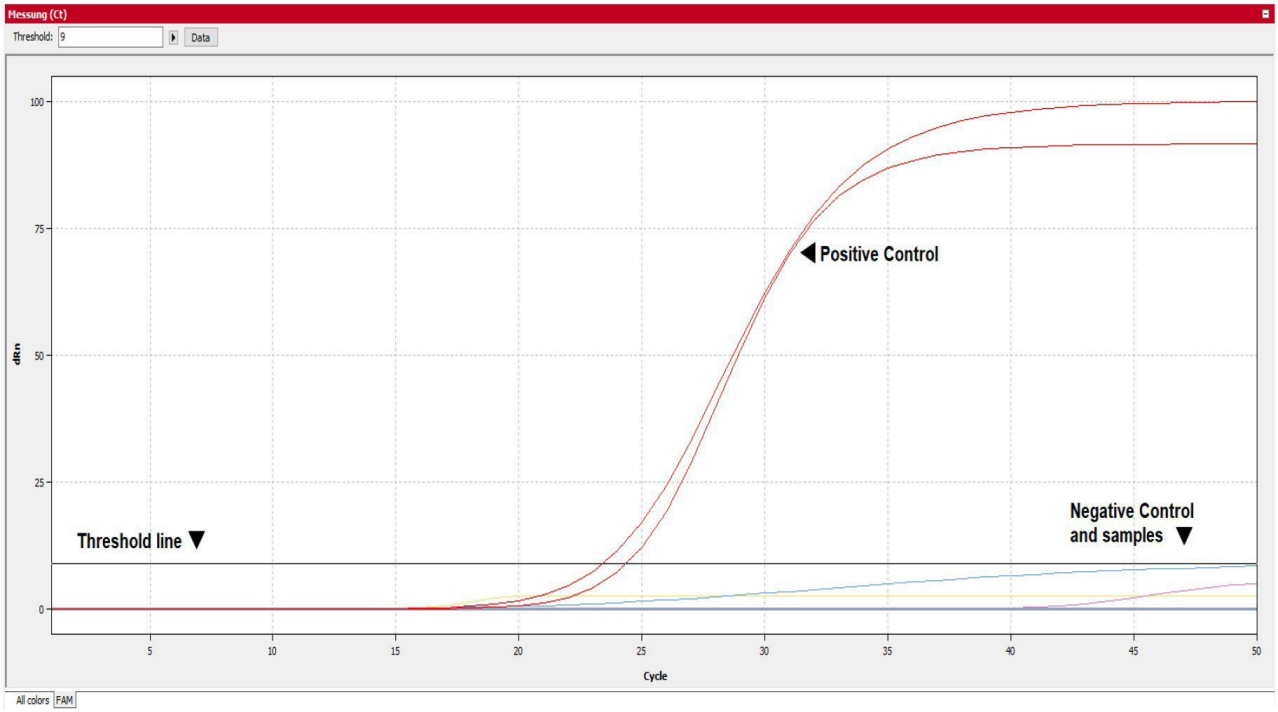

Figure 1. qPCR Amplification Curve. A total of 115 blood samples collected from the slaughtered pigs in various Class A and AA municipal slaughterhouses, which cater to backyard farms only, in 8 provinces in Luzon Island, Philippines, were tested for the presence of *B. suis* DNA using qPCR assay. There was no observed increase in fluorescence in the negative control and blood samples tested, indicating that *B. suis* DNA was not detected in all blood samples. Only the positive control increased fluorescence indicating the validity of the test run of the samples.

Table 2. Incidence of swine brucellosis according to provinces and slaughterhouses by ELISA and Real- time PCR

VARIABLE	ELISA			Real-Time PCR		
	N	N Positive	Positive (%)	N	N Positive	Positive (%)
PROVINCES						
Cavite	18	0	0	18	0	0
Laguna	13	0	0	13	0	0
Batangas	11	0	0	11	0	0
Nueva Ecija	15	0	0	15	0	0
Camarines Sur	14	0	0	14	0	0
Benguet	13	0	0	13	0	0
Occidental Mindoro	16	0	0	16	0	0
Pampanga	15	0	0	15	0	0
All animals	115	0	0	115	0	0

4. Discussion

Swine brucellosis caused by the *B. suis* is an endemic disease that has been reported in various countries across the continent, including Asia (Cambodia has prevalence rate of 0.2% [15], Bangladesh has 6.7% [14], Nepal has 3.9% [25], India has 41.04%) [26], Africa (Cameroon has 0.19% [27], Egypt has 4.83% [28], Kenya has 0.57% [29], Ethiopia has 4.5% [30], Congo has 4.42%) [31], Europe (Italy with 32.5% [32], Serbia has 9.4% [33], Spain has 59.3% [34], Croatia has 1.3%) [35], and North America (USA has 18%) [36]. The results of the study showed that the Philippines, specifically the Luzon Island, have 0% prevalence of *B. suis*. The animals tested were neither infected with *B. suis* nor have been previously exposed/ infected with the said pathogen (or at least exposed enough to elicit antibody response). This is in contrast with the previous studies of Topacio (1937) and Tiglao (1990) where the observed prevalence of *B. suis* were 2.2% and 17.65%, respectively. The study of Tiglao (1990) [22] involves slaughtered pigs from both backyard and commercial farms which collected samples from 13 provinces of the Philippines, including 8 provinces in Luzon such as Rizal, NCR, Pampanga, Tuguegarao, Batangas, Bulacan, Benguet, and La Union. Meanwhile, in our study, the animals involved slaughtered pigs from backyard farms in 8 provinces, namely Cavite, Laguna, Batangas, Nueva Ecija, Camarines Sur, Benguet, Occidental Mindoro, and Pampanga. The research by Tiglao (1990) [22] showed that the prevalence of *B. suis* infection in pigs was 0.44% in Benguet, 0.88% in Batangas, and 2.42% in Pampanga, which is in contrast with 0% prevalence detected in this study for all three provinces. However, the results of this study were consistent with a previous report of 0% [37] prevalence of swine brucellosis in Uganda. A 0% prevalence was also found in pigs in Nigeria [38]. The study which was reported by Staffort *et al.* (1992) [39] also confirmed that the prevalence of *B. suis* in commercial piggeries in Zambia was also 0%.

The studies reporting the occurrence of *B. suis* in the Philippines used Rapid Slide Agglutination Test for testing whereas our study employed ELISA and real-time PCR assay. To our knowledge, this is the first time that ELISA and real-time PCR assay have been used to detect the occurrence of brucellosis in pigs in the Philippines. Rapid Slide Agglutination Test reportedly has high specificity (100%) but lower sensitivity (95.6%) [40] compared to ELISA which has high specificity (100%) and high sensitivity (99.1%) [23]. Serological tests, especiallyin agglutination tests, may causecross-reactivity with other bacteria such as *Yersinia enterocolitica* O:9, *Salmonella urbana* group N, *Leptospira sp.*, *Vibrio cholerae*, *Francisella turansis,* and *Stenotrophomonas maltophilia*. Serological tests cannot differentiate responses to any species of *Brucella* with smooth LPS in the cell wall, including *B. suis, B. melitensis*,

and *B. abortus* [7]. Real-time PCR, on the other hand, was used for this study because real-time PCR has been shown to have high specificity and sensitivity for the detection of *Brucella* DNA as compared to other conventional PCR and serological tests [41]. Hansel *et al.* [24] reported that the average efficiency of *B. suis* real-time PCR is 95%. Moreover, real-time PCR can quantify the
B. suis chromosome 2 biovar 1 gene expression.

The negative results of the current study might be due to the natural resistance against brucellosis of the animals [42]. Another reason is that farmers' awareness may be better so biosecurity and disease prevention programs are implemented in the backyard swine farms, thereby reducing the risk of disease transmission, including *B. suis* in pigs. Due to the limited documentation of *B. suis* infection in the Philippines, it is difficult to determine the cause of the 0% prevalence detected in this study. In addition, the latest study on the prevalence of *B. suis* in the Philippines dates back more than three decades. Non-detection of the pathogen or exposure to the pathogen in the samples tested in this study may have been cause by a number of factors - including, but not limited to, improved overall veterinary healthcare, improved biosecurity and hygiene practices by hog-raisers and/or meat vendors, limited sample population, limited sampling coverage, etc. It is also possible that the sampling was performed outside of the peak seasonal incidence of *B. suis* - which is in itself is unknown in the Philippines due to limited sampling and reporting of the disease. It is also of note that the Philippines has notably lower prevalence rates of B. suis compared to other countries such as Spain, India, and Italy.

5. Conclusions

In conclusion, there were no *B. suis* antibodies and *B. suis* chromosome 2 biovar 1 gene detected from swine of Luzon using ELISA and real-time PCR assay. Our findings suggest the absence of the pathogen in the blood of pigs in the 8 provinces of Luzon Island. Further studies are recommended to investigate other *B. suis* stains, increase the study population size and coverage of sampled areas across the Philippines.

Availability of Data and Material

Readers can access all datasets that support conclusions.

Author Contributions

Conceptualization, C.C., C.P.F.C.; Methodology and Investigation - C.C., C.P.F.C., S.E.A.S, A.P.R.S and M.S.E.G.L.; Writing – Original Draft, C.C., Writing – Review & Editing, C.C., C.P.F.C., Resources, C.C., C.P.F.C.; Supervision, C.P.F.C., S.E.A.S, and A.P.R.S.

Ethics Approval and Consent to Participate

This study utilized blood samples collected by another project with IACUC Permit UPLB 2022-01, entitled "Molecular Detection and Serological Profiling of Swine Influenza and Classical Swine Fever in Backyard Farms in the Philippines" which was headed by Dr. Lola (co-author) during the sample collection.

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Conflict of Interest

The authors declare no conflict of interest.

Abbreviations

B. abortus, Brucella abortus; B. melitensis, Brucella melitensis; B. suis, *Brucella suis*; CFT, complement fixation test; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent

assay; FPA, fluorescence polarization assay; LPS, lipopolysaccharide; RBPT, rose bengal plate test; real-time PCR, real-time polymerase chain reaction.

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