RESEARCH ARTICLE

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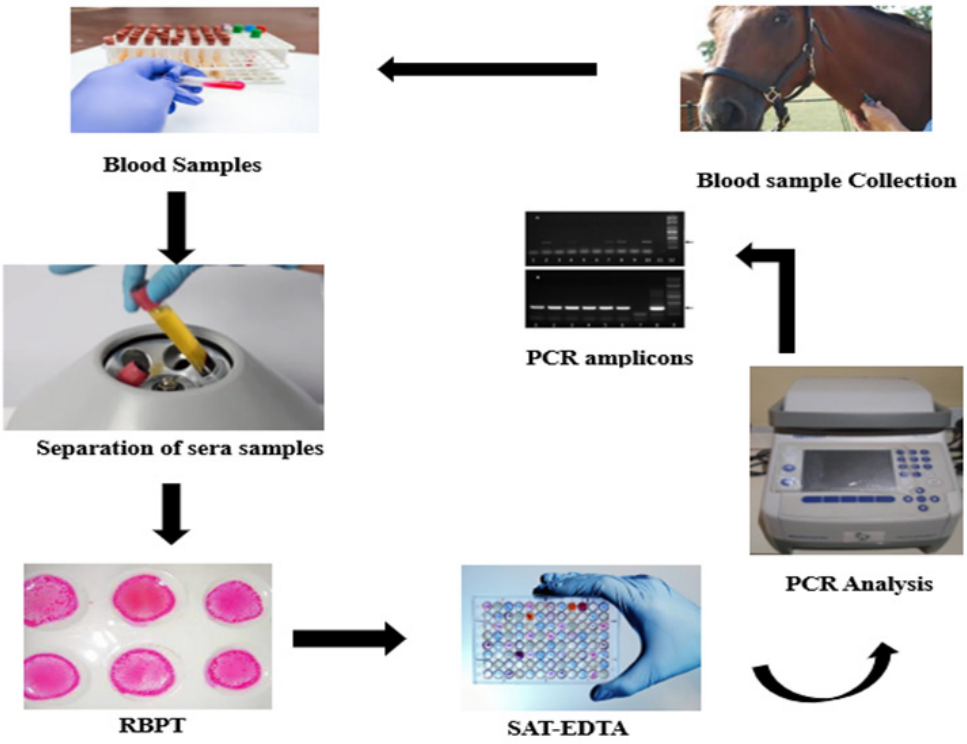

Highlights

- High prevalence of *Brucella abortus* among horses in Kano Metropolis.
- The distribution of *B. abortus* infection among test horses is shown to be more densely distributed in Gwale, Nassarawa and Kano Muncipal Council.
- Range values from 5.21-6.60 were found in Nassarawa, Kano Municipal Council and Gwale LGAs.
- The study combined two specific antibody detection methods and a molecular detection method (AMOS-PCR).

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MOLECULAR DETECTION AND GEOSPATIAL DISTRIBUTION OF *BRUCELLA* INFECTIONS IN HORSES IN KANO METROPOLIS, KANO STATE, NIGERIA

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Abstract: Brucellosis is a major zoonotic disease that affects several domestic, wild and marine mammals, and man. It causes serious economic damage in livestock productivity, especially in developing countries, including Nigeria. The study aimed to determine the species of *Brucella* that infect horses and establish the spatial distribution of the infection in Kano, Nigeria. A total of 328 blood samples were collected from horses. Sera were collected from the samples and kept at -20 ^{0}C until tested. The sera were successively subjected to RBPT, SAT-EDTA and multiplex-PCR. Seventy-nine serum samples (24.0%) were positive for *Brucella* antibodies on RBPT. Out of the RBPT positive samples, 39 (49.4%) were also positive for SAT-EDTA. There was no difference between the results of the two tests (RBPT: p=0.4420; SAT-EDTA: p=0.124). Of the 39 SAT-EDTA positive sera, 32 (82.1%) were confirmed to be *Brucella abortus*. Fifteen of the 16 mares tested were positive for *B. abortus* while 73.9% of the stallion were positive. All horses in the age groups of 1-5 and 11-15 years were 100% positive, while 72.0% of the horses in the 6-10 year age group were also positive. The study showed 81.8 % detection rate of *B. abortus* using AMOS-PCR and that 15 (93.7%) of the female horses tested (16) had *B. abortus* while 17 (73.9%) of the 23 male horses tested had *B. abortus.*

***Keywords*:** Brucellosis, RBPT; SAT-EDTA and multiplex-PCR; zoonotic diseases

INTRODUCTION

Brucellosis is one of the major zoonotic diseases that affects several domestic, wild and marine mammals, and man (Godfroid *et al*., 2005; OIE, 2000; Karthik *et al*., 2016). It also causes serious economic problems in terms of livestock production and productivity, especially in developing countries. The disease has been reported worldwide with reports in almost all continents of the world (Esuruoso, 1974). The disease has been reported in Nigeria in many domestic animals like cattle, sheep, goats, horses and humans (Esuruoso, 1974; Adesiyun & Abdu, 1984; Bale & Kwanashie, 1984; Oladosu *et al*., 1986; Kudi *et al*., 1997; Ehizibolo *et al*., 2011; Kaltungo, 2013; Ardo & Abubakar, 2016; Baba, 2016; Tijjani *et al*., 2017; Buhari *et al*.,2020; Baba *et al*., 2021). The disease in horses has been reported to affect mainly the joints and bursae horses though it may also affect the reproductive system and musculoskeletal systems (Collins *et al*., 1971; OIE & Epizooties, 2019).

There are 11 *Brucella* species known to infect domestic, wild animals, marine species and man and they include *B. abortus*, *B. melitensis*, *B. ovis*, *B. suis*, *B. canis*, *B. neotomae*, *B. microti*, *B. maris, B. ceti* and *B. pinnipedialis* and *B. inopinata* which has been isolated from women breast implants (Verger *et al*., 1987; Foster *et al*., 2007; Scholz *et al*., 2008).

Different methods have been used for the diagnosis of brucellosis. However, in most cases serological tests are being employed due to the nature of the organism as its confirmation is mostly done by competent laboratories due to its zoonotic nature (Bale, 1980). Serological tests mostly employed in the diagnosis of *Brucella* infections include RBPT, SAT, SAT-EDTA, Combs test, 2-Mercaptoethanol, MRT, SPT, CFT, Buffered Antigen Test (BPAT), Lateral Flow Test and Fluorescence Polarization Assay (FPA), among others (Baba, 2016). Molecular techniques have also been used in the identification of *Brucella* species. (Fekete *et al*., 1990; Bricker & Halling, 1994; Huber *et al*., 2009; Bertu, 2014). Similarly, Ohtsuki *et al*. (2008) used loop-mediated isothermal amplification method to detect *Brucella* spp. In addition, Pan *et al*. (2011) developed and applied the novel visual loop-mediated isothermal amplification of omp25 sequence for the rapid detection of *Brucella* spp.

Serology has remained the main means for identifying infection with *Brucella* in Nigeria with less attention in identifying the actual bacterium causing the disease due to poor laboratory backup for clinicians in the field. Where laboratory backup is available, the risks accompanied by working directly in identifying the organism have made it difficult for routine confirmation of the organism. This has made the disease to spread among cattle herds and other animal species with potential consequences.

Another problem in the diagnosis of the disease due to *Brucella* infection is the poor knowledge, attitude and practices of livestock owners and even the general public,

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especially horse owners and their grooms of other animals apart from cattle. This makes them to come hand-in-hand with the disease agents unnoticed and even facilitate their spread and transmission. The poor knowledge of the actual *Brucella* species infecting horses and other domestic animals in Nigeria, especially due to the poor disease surveillance network, poor laboratory back-up and inadequate clinical services to horse owners can also allow the disease to continue to flourish among horses and others animals. In the process, the disease agents, *Brucella* species will continue to ravage the livestock industry in Nigeria, especially that the disease has been reported to be endemic in Nigeria (Esuruoso, 1974; Falade, 1974; Falade *et al*., 1975; Falade and Shonekan, 1982; Avong, 2000; Bertu *et al*., 2010; Ior *et al*., 2013). The lack of knowledge on the *Brucella* species infecting livestock and particularly horses in Nigeria and the risk of zoonosis, especially due to *B. abortus* and *B. melitensis* in humans will certainly be a cause for concern due to the serious zoonotic implications of these particular *Brucella* species.

Few studies have reported *Brucella* species infecting livestock in Nigeria. These include Bertu (2014) who demonstrated *Brucella abort*us infecting cattle under the pastoralist herds in Kaduna and Plateau states, and Buhari *et al*. (2020) identified *B. abortus* and *B. suis* in the blood of sheep and goats in institutional farms. and Kaltungo (2018a, 2018b) showed *B. melitensis* infection in camels in a slaughter slab in Zaria.

In Nigeria, horses are being widely used in leisure, marriage ceremonies, horse racing, playing polo in addition to meat and skin. These activities lead to closer contact of horses with humans and hence increase the risk of acquiring pathogens such as *Brucella.* The objectives of the study were to detect the *Brucella* species present in horses using the Polymerase Chain Reaction (PCR) technique and to determine the geographic distribution of the equine brucellosis in Kano metropolis, Kano State, Nigeria.

MATERIALS AND METHODS

The study was conducted in the Kano metropolis, Kano State, Nigeria. The State is located between latitude 13^0N and 11^0N and between longitude 8^0W and 10^0E. Administratively, Kano State has 44 Local Government Areas (LGAs), with Kano metropolis having eight out of these LGAs (Fig 1). The State has a total land mass of 20,760 Km^2 with and a human population of 9,383,682 (NPC, 2006). It also has an elevation range of about 650 m above the sea level but extend up to 1000 m above mean sea level at Rishi hills (Aliyu *et al*., 2021). The State is bounded by Jigawa State to the North and East. It shares borders with Katsina and Kaduna states from the West and Southwest, respectively. It also shares borders with Bauchi State from the extreme Southeast (Figure 1). There are an estimated 2,500 horses in Kano State out of the 1.4 million horses reported to be in Nigeria (FAO, 2019).

The horses from four of the eight LGAs in Kano metropolis were sampled. The LGAs were selected based on the purposive sampling method as most of the horses in Kano State are located within the Kano metropolis. The stables were selected based on convenience sampling method and agreement by the stable owners to participate in the study. Furthermore, the horses sampled were based on simple random sampling within the stables. The method of Michael (2005) was used to arrive at the sample size of 328 horses based on the prevalence of 20.1% for *Brucella* antibodies in horses in Kaduna state, Nigeria as reported by Baba (2016). A total of 10 ml of blood was collected aseptically

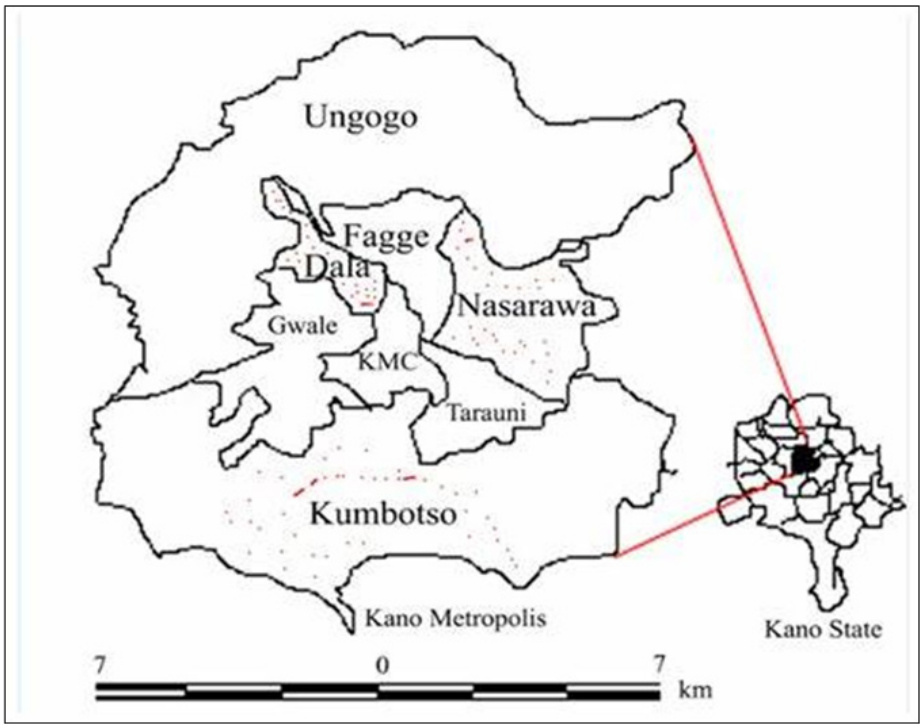

Figure 1: Map of Kano State and the Local Government Areas Sampled in Kano metropolis (A, B, C and D). Source: Department of Geography, Ahmadu Bello University, Zaria, Nigeria.

using 18 G needles and 20 ml syringes from each horse via the jugular vein after each horse was restrained by an assistant (as contained in the ethical clearance obtained from the ethical clearance committee, University of Ilorin). The blood samples were collected in 20 ml blood sampling bottles and kept in a slanting position in a Coleman box with ice. Details of each horse: location, sex, age, breed, and use, were recorded. The blood samples were taken to the Bacterial Zoonoses Laboratory, Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria where they were centrifuged at 1000g for 5 min for serum separation. The separated serum samples were then decanted into serum tubes and stored at -20 ^{0}C until used.

The coordinates (latitude and longitude) of each sample location were taken using Global Positioning System (GPS) receiver (Etrex®, Taiwan). The ArcGIS software version 10.3 and its extension package ArcView spatial Analyst (Environmental System Research Institute, Inc. Redlands, USA) were used to develop the geospatial map of *Brucella* infections in the study LGAs and Kano State as a whole.

Serum samples were screened for *Brucella* infection using Modified Rose Bengal Plate Test (mRBPT) as described by Bale (1980) and modified by Berta (2014). Serum samples that were positive for mRBPT were further screened with a Serum agglutination test with ethylene diaminotetraacetic acid (SAT-EDTA) as further evidence of actual infection. The serum samples that were positive for *Brucella* species by SAT-EDTA were used for molecular detection of the *Brucella* species using AMOSPCR (The oligonucleotide primers used included: *B. abortus*- specific (GAG-GAA-CGG-AAT-TTT-TCC-AAT-CCC), *B. melitensis*-specific (AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA), *B. ovis*-specific (CGG-GTT-CTG-GCA-CCA-TCG-TCG), *B. suis*-specific (GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG) and IS711-specific (TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT) (Bricker and Halling, 1994). Initial denaturation was at 95 ^{0}C for 15 min. This was followed by 35 cycles of denaturation at 94 ^{0}C for 30 s. Then annealing at 57 ^{0}C for 1 minand 30 s extension at 72 ^{0}C for 2 min, followed by final extension for 10 min. The amplified products were visualized in ethidium bromide-stained agarose gel (1%) electrophoresis under UV illumination.

Data Analyses

Data generated were presented in tables. The data were also analyzed using SPSS version 17.0 (2010). Fisher's exact test was used to test for agreement between various tests such as; M-RBPT, SAT-EDTA, 2-ME, and PCR. P-value <0.05 was considered significant.

RESULTS

A total of 79 (24.1%) out of the 328 serum samples were positive for *Brucella* antibodies using mRBPT. Furthermore, of the 79 positive samples, 39 (49.4%) were further positive for *Brucella* antibodies using SAT-EDTA (Table 1). There was no statistically significant difference in the seroprevalence by test used (RBPT: Pearson Chi-Square= 2.821[a], p= 0.420; SAT-EDTA: Pearson Chi-Square= 5.763[a], p= 0.124). Of the 39 serum samples tested for characterization of *Brucella* species, 32 (82.1%) were confirmed to be *Brucella abortus* positive (Table 2; Plate I). The infection of *B. abortus* between male and female horses is shown in Table 3. Of the 39 serum sampled tested 16 were from female horses while the remaining 23 were from male horses. Of which 15 females (93.8%) were confirmed to have *B. abortus*. Similarly, of the 23 male horses, 17 (73.9%) were positive for *B. abortus* while the remaining 6 (26.1%) were negative for *.B abortus* (Table 3). There was no statistically significant difference in the identification of *B. abortus* by sex (Fisher's Exact Test= 4.623; p= 0.150).

The identification of *Brucella abortus* by age is presented in Table 4. All the 9 (100%) horses in the age group 1 to 5 years old were positive for *B. abortus* while of the 25 horses in the 6 to 10 years old bracket 18 (72%) were positive for *B. abortus*. Similarly, all the 4 (100%) horses in the 11 to 15 years old bracket and were positive for *B. abortus* (Table 4). There was no statistically significant difference in the identification of *B. abortus* by age (Fisher's Exact Test= 2.824; p= 0.260).

Among the horse breeds under the study, Sudan breed of horses recorded the highest number {15 (100%)} of horses positive for *B. abortus* using AMOS PCR followed by Arewa and Talon breeds of horses with 12 (66.7%) and 5 (83.3%) having *B. abortus* respectively and least by Argentine breed of horses with no trace of *B. abortus* antigen detected in their sera (Table 5). There was no statistically significant difference in the identification of *B. abortus* by breed (Fisher's Exact Test= 3.624; p= 0.160). Considering the *Brucella* species identified by use of horses, the study

Table 1: Seroprevalence of *Brucella* infection using RBPT and SAT-EDTA in horses sampled in four Local Government Areas (LGAs) of Kano Metropolis, Kano State, Nigeria.

LGA	Number of Samples tested	Number (%) Positive by test type	
		RBPT (%)	SAT-EDTA (%)
Kano Municipal	92	28 (30.4)	17 (60.7)
Gwale	37	8 (21.6)	4 (50.0)
Nasarawa	129	28 (21.7)	13 (46.4)
Tarauni	70	15 (21.4)	5 (33.3)
Total	**328**	**79 (24.1)**	**39 (49.4)**

RBPT: Pearson Chi-Square= 2.821[a], p= 0.420
SAT-EDTA: Pearson Chi-Square= 5.763[a], p= 0.124

found that the highest identification of *B. abortus* was in polo horses as 95.45% followed by ceremonial horses with 64.71% and none in racing horses (Table 6). There was no statistically significant difference in the identification of *B. abortus* by use (Fisher's Exact Test= 1.824; p= 0.360).

The distribution of *B. abortus* using geospatial technique, within the sampling locations and the LGAs as a whole is presented in Figures 2 and 3 respectively which implies that there was geographical/spatial variation within the LGAs and between the LGAs where the horses were sampled. The prevalence decrease outward from the point of sampling. The distribution is most concentrated in Nasarawa LGA with 14.21% to 17.00% within the immediate vicinity of the sampling location and reduced as one moved away from the location. The least distribution was in Tarauni LGA with 3 to 5.8%.

Table 2: Detection of *Brucella* species infecting horses by sex using AMOSPCR from four selected Local Government Areas (LGAs) of Kano state.

LGA	No. of sera Tested	No. positive for *B. abortus*
Kano Municipal	17	11 (64.70)
Gwale	4	4 (100.00)
Nasarawa	13	12 (92.31)
Tarauni	5	5 (100.00)
Total	**39**	**32 (82.05%)**

SAT-EDTA: Pearson Chi-Square= 5. 763[a], P= 0.124

Table 3: Detection of *Brucella* species infecting horses by sex using AMOSPCR from four selected Local Government Areas of Kano state.

LGA	Sex			
	Female		Male	
	No. of sera Tested	No. positive for *B. abortus*	No. of sera Tested	No. positive for *B. abortus*
Kano Municipal	0	0 (0.0)	17	11 (64.7)
Gwale	4	4 (100.0)	0	0 (0.0)
Nasarawa	8	7 (87.50)	5	5 (100.0)
Tarauni	4	4 (100.0)	1	1 (100.0)
Total	**16**	**15 (93.8)**	**23**	**17 (73.9)**

Fisher's Exact Test= 4.623; p= 0.150

Table 4: Detection of *Brucella* species using the AMOS PCR from serum of sampled horses by age in four LGAs of Kano Metropolis, Kano State, Nigeria.

Age	No. of sera Tested	No. positive for *B. abortus*
1 – 5 years	9	9 (100.0)
6 – 10 years	25	18 (72.0)
11 – 15 years	4	4 (100.0)
>15 years	1	1 (100.0)
Total	**39**	**32 (64.7)**

Fisher's Exact Test= 2.824; p= 0.260

Table 5: Detection of *Brucella* species using the AMOS PCR from serum of sampled horses by breed in four LGAs of Kano Metropolis, Kano State, Nigeria.

Breed	No. of sera Tested	No. positive for *B. abortus*	No. negative for *B. abortus*
Arewa	18	12(66.67)	6 (33.33)
Sudan	15	15 (100.00)	0 (0.00)
Talo	6	5(83.33)	1 (16.67)
Total	**39**	**32 (66.67)**	**7 (33.33)**

Fisher's Exact Test= 3.624; p= 0.160

Table 6: Detection of *Brucella* species using AMOS PCR from serum of sampled horses by use in Kano Metropolis, Kano State, Nigeria.

Uses	No. of sera Tested	No. positive for *B. abortus*
Ceremonial	17	11 (64.71)
Polo	22	21 (95.45)
Total	**39**	**32**

Fisher's Exact Test= 1.824; p= 0.360

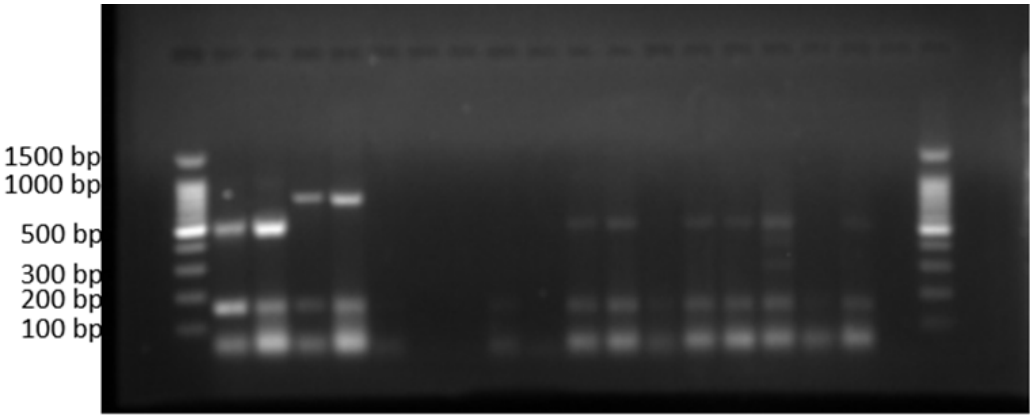

Plate I: PCR amplicons obtained in AMOS-PCR on serum samples. Lane M- 100 bp DNA marker; Lane PA- *Brucella abortus* positive control (498 bp); Lane PM- *B. melitensis* positive (731 bp) control; Lane 2– *B. abortus* positive samples; Lanes 6,7,9,10,11, and 13. Lanes 1, 2, 3, 4, 5, 8, and 12- negative samples; Lane NC- Negative control; Lane M- 100 bp DNAmarker.

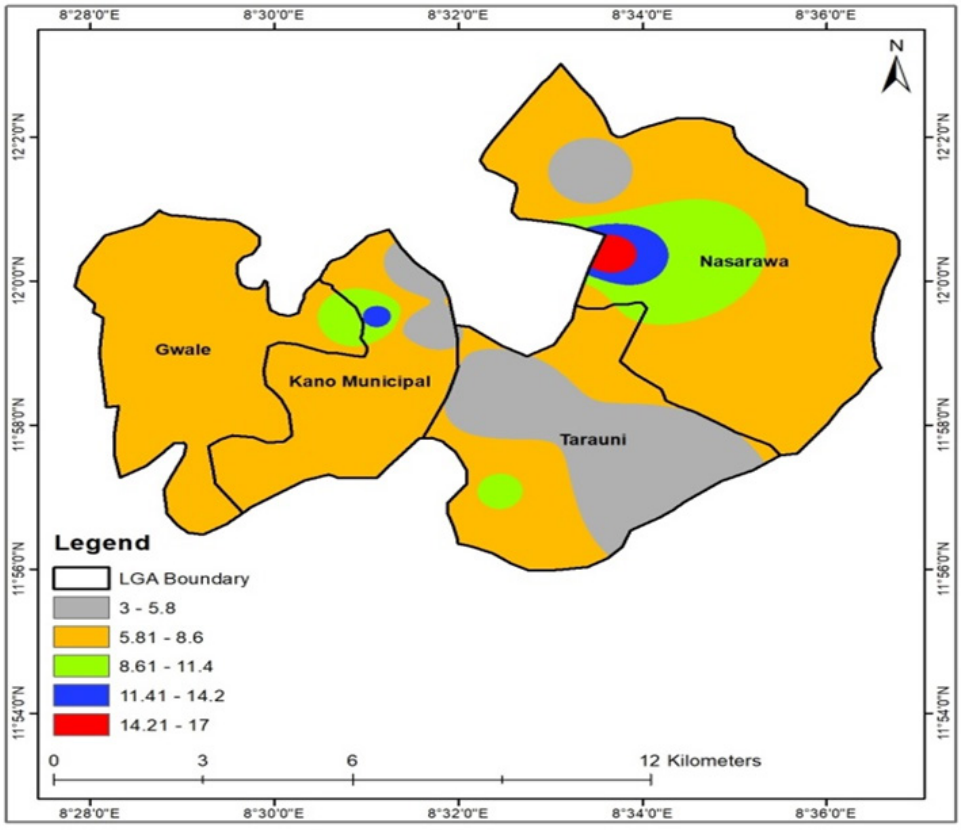

Figure 2: Spatial Interpolation map imagery showing *Brucella abortus* density distribution using RBPT in the LGAs of Kano metropolis, Kano State, Nigeria.

DISCUSSION

From the study, the horses encountered during the study included Arewa, Argentine, Sudan and Talon breeds of horses. This has confirmed the presence of exotic breeds of horses in Nigeria. Such report has also been made by Fadimu (2014) and Baba (2016). Many workers like Kaltungo (2013) and Buhari (2014) have reported higher seroprevalence rates with SAT-EDTA than with RBPT with statistically significant differences.

The finding of seroprevalence of *Brucella* among the sampled horses indicates that these horses might have been mixing with other domestic animals within the area as Kaltungo (2018a, 2018b) and Buhari *et al*. (2020) have reported cattle, small ruminants and camels meeting at grazing and watering points and that such mixing might have facilitated transmission and spread of infections in these animal species. The fact that these horses had seroprevalence of *Brucella* means that there is high risk of infection by the grooms and even the horse owners who may ride them once in a while. Another implication is that, at least for the polo and racing horses, they are usually involved in tournaments and their involvement in tournaments may mean spread of the infection to other distant areas. In another study in Kano State, Fadimu (2014) reported seroprevalence of 5.3% and 4.3% by using RBPT and SAT respectively. This study has further confirmed the existence of *Brucella* infection in horses in the State. The finding of higher seroprevalence in this study might be that different horses might have been used by the two researchers. Other reports of *Brucella* infection in Nigeria include those of Esuruoso (1974); Bale & Kwanashie (1984); Ehizibolo *et al*. (2011); Bertu (2014); Baba (2016), and Yakubu (2016). A report of *Brucella* seroprevalence in rodents in Nigeria has similarly earlier on been made (Avong, 2000).

The identification of *B. abortus* organisms through the use of AMOS-PCR in the blood of horses in this study means that the organisms are circulating in these horses. Thus, there is active *Brucella* infection in these horses. The implication of this is that the grooms and even their owners stand the risk of contracting *Brucella* infection anytime they initiate blood oozing out of the body of these horses. This is possible through injury of the mouths of the horses with bridle, injury of the body of the horse through the use of coarse grooming tools and such other materials that can initiate tear of the skin. Bertu (2014) and Buhari *et al*. (2020), both in Nigeria, also reported demonstrating *B. abortus* in the blood of cattle, sheep and goats during their studies. In addition, Buhari *et al*. (2020) also reported detecting *B. suis* in sheep and goats blood samples. The existence of *Brucella* species in domestic animals means serious public health hazards and could be as a result of poor disease surveillance, and control strategies in place. It could also be due to the poor laboratory back up along with the relatively few Veterinary personnel that could cover the vast country to provide veterinary health care services. The poor public health services along with inadequate laboratory back up at rural areas where most of the animals are found might also result in the disease, brucellosis, being in humans without notice. Many cases in human and even veterinary clinics are routinely diagnosed based on clinical signs and symptoms. The lack of adequate public and to a certain extent private medical and veterinary care services could similarly result in many cases of human brucellosis without due attention being made for proper diagnosis. The cases routinely diagnosed in many human hospitals and clinics could actually be brucellosis as no real attention is routinely given for proper laboratory diagnosis of cases.

The identification of *B. abortus* was higher in female horses (93.7%) than in males (73.9%) though this was not statistically significant. None the less the chances of the spread of the infection could be said to be similar since both sexes are being used. The detection of *B. abortus* in horses by age indicated that all age groups were significantly involved with the organisms. Thus, the risk by the owners and grooms, no matter which age of horse is used is still there. The implication here is that youths, who drive pleasure in riding horses at weekends run the risk of becoming infected, especially that young horses are more involved in these weekend ride. The same inference can be deduced for identification of *B. abortus* by breed and use of horses since the horses are continuously used and there are chances for the horses to mix during durbar, weekend ride, polo or racing tournaments. Earlier, Jalil (2008) has reported human brucellosis acquired through horses and that the risk is real. The exchange of grooming tools by grooms as reported by Baba, (2016) could also lead to the spread of the disease, should any of the horses groomed be infected with *Brucella* species and has bruises or tears on the skin.

The documentation of geospatial distribution of *B. abortus* infection in horses in the study area as produced in this study seems to be the first of such documentation to the best of our knowledge in the area. Similar documentations of the distribution of *Brucella* infection in animals in Nigeria were those by Bertu, 2014; Kaltungo, (2018a) and Buhari *et al*. (2020) who documented the distribution of *Brucella* infections in cattle and small ruminants in Kaduna, Katsina, Plateau and Sokoto States, Nigeria.

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DECLARATION OF CONFLICT OF INTEREST

There is no conflict of interest as all researchers worked as a team and sought for permission from their employers for the conduction of the research project. The researchers also unanimously agreed to publish the work in this journal.

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