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Molecular Survey of *Brucella* Species in Sheep and Goats Slaughtered at Sokoto Modern Abattoir, Sokoto State, Nigeria

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ABSTRACT: *Brucellosis is an important zoonotic disease affecting animals and humans. A cross-sectional study was conducted to determine the prevalence of brucellosis in sheep and goats slaughtered at Sokoto metropolitan abattoir using serological assay and molecular detection technique. Serum samples from 131 sheep and 242 goats were analyzed. An overall prevalence of 10.72% (40/373) was recorded using PCR while none of the samples was positive by RBPT. The prevalence rates in sheep and goats were 12.2% (16/131) and 9.91% (24/242) respectively, while the sex prevalence rates were 16.47% and 4.54% for rams and ewes respectively. The prevalence based on breed were 15.18%, 11.11% and 0% for Ouda, Balami and Yankasa respectively. The sex prevalence for goats were 10.08% and 9.75% for bucks and does, while the breed prevalence rates were 50%, 33.3% and 9.28% for Sahelian, West African Dwarf and Red Sokoto goat respectively. A statistically significant association between Brucella infection and sex of sheep (p=0.037) was observed. However, there was no statistically significant association (p>0.05) between the infection and the small ruminant species, breeds and sex of goats. The study revealed presence of brucellosis in sheep and goats in the study area and the occupational hazards associated with the zoonotic disease.*

Keywords: *Abattoir, Brucellosis, Goat, Molecular, Sheep, Sokoto, Survey*
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INTRODUCTION

Brucellosis is an economically important bacterial disease of livestock caused by *Brucella species*. The disease affects a wide range of animal species including cattle, sheep, goats, chickens, camels and horses. The bacterium has also been isolated in marine mammals and wild rodents. There are about 12 *Brucella species* reported in animals among which 6 have been reported to be zoonotic, namely *B. abortus* in cattle, *B. melitensis* in sheep and goats, *B. canis* in dogs, *B. suis* in pigs, *B. ceti* and *B. pinnipedalis* in marine mammals (Foster *et al*.,

2007). The disease has a limited geographic distribution, but remains a major problem in the Mediterranean region, Southern Europe, West and central Asia, South America and Africa (Nielsen and Duncan, 1990; Corbel, 1997; Godfroid *et al*., 2005), with considerable variation between herds, and between areas and countries. The disease is transmitted from animals to humans by consumption of unpasteurized milk and dairy products, consumption of under-cooked meat or occupational exposure usually resulting from direct contact with infected animals (Peck and Bruce, 2017; Craighead *et al*., 2018). Bucellosis is an economically important disease in livestock which has been associated with abortion, decreased productivity and loss in trade (CFSPH, 2018). In accessing the economic impact of brucellosis in case of bioterrorist attack it will have an economic impact of $477.7 million per 100,000 persons exposed (Bamaiyi *et al*., 2014). In Malaysia, the annual economic impact due to brucellosis in small ruminants was reported at almost US$2.6 million (Bamaiyi *et al*., 2015).

Brucellosis is highly endemic in developing nations and remains a global burden with more than 500,000 cases in both animals and humans every year (Wafa, 2018). As few as 10 to 100 organisms can cause the disease in humans which is associated with severe acute disease and is recorded as endemic in several countries (Smith and Shorman, 2019).

Brucella is mainly transmitted via ingestion of food or water contaminated with secretions or aborted fetal remains from infected animals. In goats, *brucella* excretion in vaginal discharge is lengthily profuse and its shedding continues for some months while in sheep, excretion is not lengthy and cessation occurs within few weeks after abortion or full-term parturition, but kids and lambs that nurse from infected dams may shed *B. melitensis* in their feces (Tilahun *et al*., 2016). The risk factors for infection include handling of infected animals (tissues and body fluids such as blood, urine, vaginal discharges, aborted animal foetuses and especially placentae), ingestion of contaminated animal products such as meat, raw or unpasteurised milk and milk products (Bamaiyi, 2016). Serum is the sample of choice for PCR technique used as a good source of DNA due to its anticoagulant and hemoglobin free nature (Khan and Zahoor, 2018). *Brucella spp* in contrast to other non-spore forming pathogenic bacteria lasts for long outside mammalian hosts under favorable conditions. In several studies stated, *Brucella* can survive for about 4 h 30minutes at temperature lower than 31°C though it is sensitive to sunlight (WHO, 2006).

The aim of the study was to investigate the presence of *Brucella* species and its antibodies, establish the prevalence of *Brucella* antigen in sheep and goats slaughtered at Sokoto metropolitan abattoir using RBPT and Polymerase chain reaction (PCR) respectively. In addition, to ascertain possible association of the infection with species, sex and breeds of sheep and goats.

MATERIALS AND METHODS

Study area

The study was conducted at the Sokoto modern abattoir located in Sokoto North Local Government Area Sokoto State, Nigeria. The Abattoir is located between longitudes 005° E and 13.416'E and latitudes 13°N 04.209'N (Figure 1). A cross-sectional study was conducted and systematic random sampling technique was employed. For this study, samples were collected from every 3rd animal, which sums to a total of 15-20 samples daily. A total of 373 samples were collected, 131 and 242 from sheep and goats respectively.

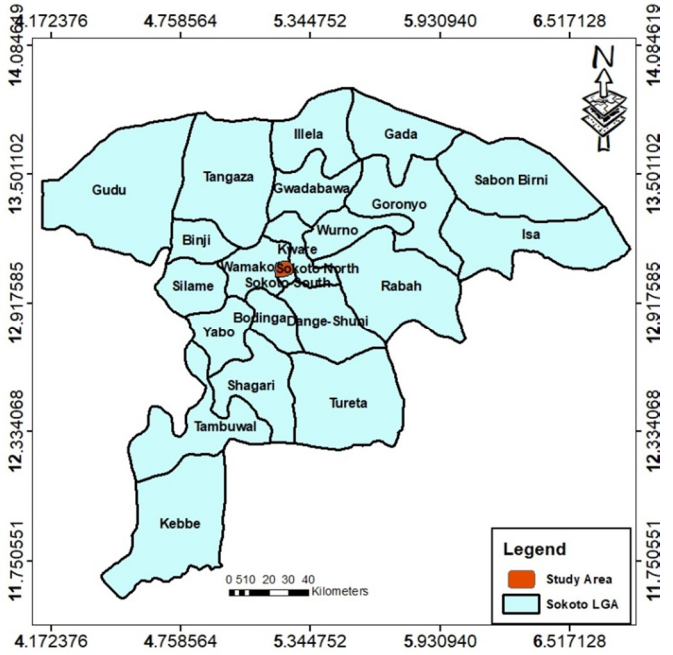

Figure 1: Map of Sokoto State showing the 23 LGA's of the State and the Study Area

Sample collection

Relevant information about each animal such as sex, breed and species were collected. Following proper restraint, 5ml of blood sample was aseptically collected from the jugular vein before slaughter using a sterile vacutainer (anti-coagulant free). Each sample was properly labeled and transported immediately in an ice chest to the Central Research Laboratory, Faculty of Veterinary Medicine, Usmanu Danfodiyo University

Sokoto. Sera samples were harvested by centrifugation at 3000g for 10-15 minutes. The samples were transferred into 1.5ml microfuge tubes (eppendorf®) and stored at -20°C until required.

Laboratory Analyses

Serological Assay (Rose Bengal Plate Test)

The test assay was conducted as per technique described by Alton *et al.* (1988). The RBPT antigens were sourced from IDvet® France and stored at +2°C ≤ +8°C. Equal volume of antigen and serum (30µl) was placed on a glass slide, stirred using toothpick then agitated for four minutes. Appearance of agglutination indicated positive sample whereas absence of agglutination indicates a negative sample.

Molecular detection

DNA Extraction

Boiling method was used to extract DNA from the sera samples as described by Queipo-Ortuno *et al.* (2008). The sera samples were transferred into a microfuge tube and were centrifuged at 15000g for 15 minutes. The supernatant was discarded and the pellets were suspended with 200µl of molecular grade water. The suspension was also centrifuged at 15000g for 15 minutes and the supernatant was discarded while the pellets were re-suspended in 40µl of molecular grade water. The mixture was heated in a water bath at 95 °C for 30minutes before placing it on ice for 10 minutes. The recovered DNA template was centrifuged at 15000g for 10 seconds and the supernatant was used for polymerase chain reaction.

Polymerase Chain Reaction (PCR)

PCR was done using Top Taq PCR Mastermix kit (Qiagen®), which was sourced from Malaysia. A 25µl reaction mixture was prepared comprising, 12.5µl TopTaq Master Mix 2x, 3µl molecular grade water, 5µl of DNA template and 1µl of *Brucella* genus specific forward and 1µl of *Brucella* genus specific reverse primers amplifying the 151bp of the bscp-31 gene (Shell *et al.*, 2017), the primers were sourced from Integrated DNA Technologies, Singapore (IDT®). The reaction was subjected to an initial denaturation at 94 °C for 5 minutes and then 35 cycles of amplification (60 seconds denaturation at 94°C, 60 seconds annealing at 60°C, 60 seconds extension at 72°C) followed by 10 minutes of final extension at 72°C. Molecular grade water and *Brucella abortus* S19 obtained from NVRI, Vom were used as negative and positive controls respectively.

Agarose Gel Electrophoresis

A 1.5% agarose gel was prepared by suspending 1.05 grams of agarose powder in 70ml of 1X Tris-Boric-EDTA (TBE) buffer and heated on a hot plate until completely dissolved. Ethidium bromide (7µl) was added into the liquid agarose before pouring into a gel caster set and allowed to solidify at room temperature. The caster was rightly placed into an electrophoresis tank and flooded with 1XTBE buffer to the maximum level before carefully removing the comb. The PCR products (8µl each) were then loaded into the wells using a 10µl Eppendorf pipette. Five microliter (5µl) of 100bp ladder (BioLabs Inc. New England) was also loaded into one of the wells before connecting the tank to a power pack and plugged to the main supply. The products were electrophoresed at 60 volts for 55 minutes. Immediately after electrophoresis, the agarose gel was viewed using a Gel Doc™ (BioRad). The gel image was captured and labelled accordingly (Table 1).

Table 1: Sequences of oligonucleotide primer used for PCR (Probert *et al.*, 2004; Shell *et al.*, 2017).

Primer	Sequence (5' to 3')	Band Size
bcsp-31 (Forward)	GCT-CGG-TTG-CCA-ATA-TCA-ATG-C	151bp
bcsp-31 (Reverse)	GGG-TAA-AGC-GTC-GCC-AGA-AG	

Data presentation and statistical analysis

Data collected from this study were entered and stored in Microsoft Excel® 2010. Prevalence rate was calculated as the ratio of positive samples to the total number of samples collected. Chi-square and logistic regression as appropriate in order to determine any association between the variables (Species, Sex and breeds) and brucellosis (*Brucella* infection). SPSS® statistical package version 20 was used for the analysis. Probability value of less than 0.05 ($p<0.05$) was considered statistically significant at 95% confidence interval.

RESULTS

A total of 373 sera comprising 131 sheep and 242 goats were analyzed using the Rose Bengal Plate Test (RBPT). However, none of the sera samples was positive, hence zero prevalence was recorded. The same 373 sera were subjected to PCR and forty sera were positive for the *Brucella* genus specific PCR with an overall prevalence of 10.72% (40/373) (Figure 2). The species-specific prevalence of *Brucella* species infection in sheep using PCR was 12.2% (16/131) which was higher than the prevalence of 9.9% (24/242) for goats. The chi-square test statistic showed no statistically significant association between *Brucella* infection and the livestock species (p = 0.494) (Table 2).

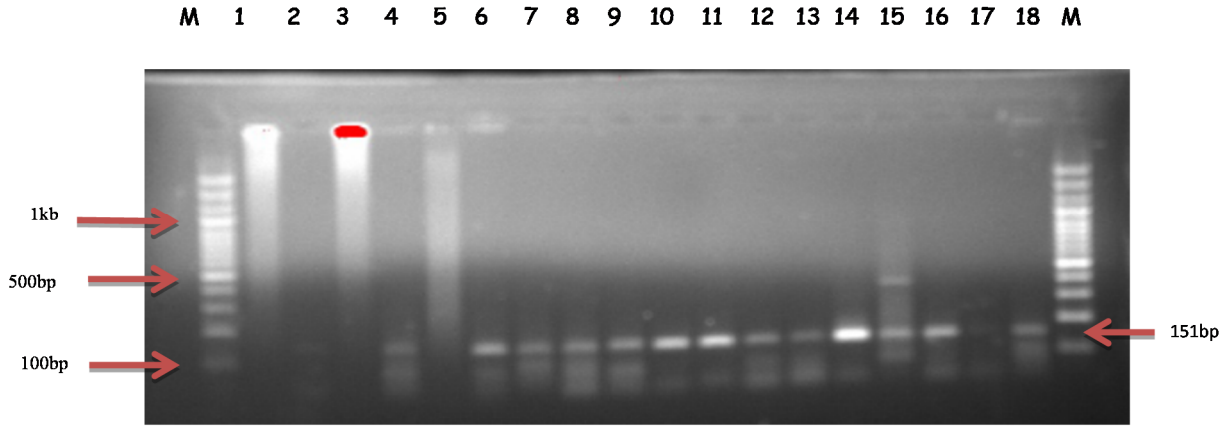

Figure 2: Representative Agarose gel electrophoresis showing positive samples using bcsp-31 gene-based PCR
M; Molecular marker (100 bp ladder), Lanes 1-8; positive samples for goat, Lanes 9-16; positive samples for sheep, Lane 17; negative control (RNAse free water), Lane 18; positive control (S19 *B. abortus* vaccine)

Table 2: Association between animal species and detection of *Brucella* species using PCR.

Species	Number of Animals	Prevalence (%)
Sheep	131	16(12.2)
Goat	242	24(9.91)
Total	373	40(10.72)

χ^2 =0.468; df =1; *p*=0.494

Table 3: Prevalence of *Brucella* species in Sheep based on sex using PCR

Sex	Number of Animals	Prevalence (%)
Ram	85	14 (16.47)
Ewe	46	2 (4.54)
Total	131	16 (12.2)

df= 1; p=0.050 By Fisher's Exact Test

Table 4: Prevalence of *Brucella* species Infection in Sheep based on breed using PCR.

Breed	Number Animals	Prevalence (%)
Ouda	79	12 (15.18)
Balami	36	4 (11.11)
Yankasa	16	0 (00.00)
Total	131	16 (12.2)

p=0.773 By Fisher's Exact Test

The sex specific prevalence of *Brucella* infection in sheep revealed that rams had a higher prevalence of 16.47% (14/85), while ewes had 4.54% (2/46). Chi square test showed statistically significant association between *Brucella* infection and sex of sheep (*p*= 0.050) (Table 3). The breed specific prevalence of *Brucella* infection in sheep revealed that Ouda had the highest prevalence of 15.18% (12/79), while Balami had 11.11% (4/36) and 'Yankasa had zero prevalence. There was no statistically significant association between breeds of sheep and *Brucella* infection (*p*= 0.773) (Table 4). The sex specific prevalence of *Brucella* infection in goats showed that bucks had a slightly higher prevalence of 10.08% (12/119) while does had 9.75% (12/123). The chi square test statistic showed no statistically significant association between *Brucella* infection and sex of goats (*p*=0.932) (Table 5). The breed specific prevalence of *Brucella* infection in goats revealed that the Sahelian breed had higher prevalence of 50% (1/2), West African dwarf 33.3% (1/3) and Red Sokoto goat had 9.28% (22/237). Logistics regression showed no statistically significant association between *Brucella* infection and the breeds of

Table 5: Prevalence of *Brucella* species Infection in Goats based on sex using PCR

Sex	Number of Animals	Prevalence (%)
Buck	119	12 (10.08)
Doe	123	12 (9.75)
Total	242	24 (9.91)

$\chi^2 = 0.007$; df=1; $p = 0.932$

Table 6: Prevalence of *Brucella* species Infection in Goats based on breed using PCR.

Breed	Prevalence (%)	p-value	Odds Ratio	95% CI
RSG	9.28 (22/237)	REF	N.A	N.A
SHL	50 (1/2)	0.111	0.102	0.006-1.693
WAD	33.3 (1/3)	0.203	0.205	0.018-2.348

CI- Confidence Interval, N.A- Not applicable, RSG- Red Sokoto Goat, REF-Reference category, SHL-Sahelian, WAD- West African Dwarf

goats (Table 6).

DISCUSSION

Brucellosis is one of the most important livestock diseases reported to be endemic in Nigeria. However, in this study none of the sheep and goats was positive for RBPT. Thus, there was 0% seroprevalence. This finding is similar to the results of Ogugua *et al.* (2015) who also recorded 0% prevalence using RBPT in Sokoto, Cadmus *et al.* (2006) in Ibadan who reported 0.86% and 0% in goats and sheep respectively, Sarker *et al.* (2016) who reported 0% prevalence in sheep and goats in Bangladesh. Moreover, Fensterbank *et al.* (1982) reported that RBPT shows poor ability to detect brucellosis in newly infected cases. Another reason which could explain the results in our study is that; in an experimental study by Gao *et al.* (2015), after experimentally infecting sheep with *B. melitensis* 16M they were euthanized at days interval post inoculation, the results he obtained of the agglutinating antibodies showed that anti-brucella antibodies began to produce at 7days post inoculation (dpi) peaked at 15dpi and gradually declined until lower detection levels were detected at 180dpi. The overall prevalence recorded in this study using bcsp-31 gene-based PCR was 10.72%.

In Nigeria, there is dearth of information on molecular detection of *Brucella* species in animals. However, there are several reports on serological detection of antibodies against the bacterial pathogen. Although, *Brucella abortus* biovar was detected in all the positive samples collected from cattle, sheep and horses (Ocholi *et al.*, 2004). In Egypt, Salama *et al.* (2011) reported 39%, prevalence using blood samples to detect *Brucella* from ovine by PCR. The result is higher than the prevalence recorded in our study which could be as a result of high level and/or endemicity of brucellosis recorded in Egypt as reported by Hegazy *et al.* (2011), Jennings *et al.* (2007) reported Egypt being one of the countries with high rates of human brucellosis. Moreover, the prevalence recorded in this study using PCR is lower than seroprevalence rates recorded in previous studies about a decade. This indicates brucellosis is still prevalent in sheep and goats in Sokoto. It could be due to the fact that they are not included in vaccination programs as reported by Junaidu *et al.* (2010), also the intermingling of different species of animals together enhancing the spread of disease between animals as well as zoonotic transmission, with transhumance practiced by Fulani herdsmen these are all important factors for endemicity of the disease (Ocholi *et al.*, 2004). In addition, a study in Iran conducted by Khamesipour *et al.* (2013) using qPCR a prevalence of 31.11% was reported in sheep, which is also higher than the finding in this study. This could be due to the higher prevalence of brucellosis seen in Iran and countries near the East region (Refai, 2002). Similarly, the prevalence of 9.91% recorded for goats in this study is lower than the report of Al-Garadi *et al.* (2011) who recorded a prevalence of 29.5% in goats in Malaysia using qPCR. This could be attributed to the high endemicity of brucellosis in Southeast Asian countries (Bamaiyi *et al.*, 2014) and the unstable livestock population due to importation and exportation of animals, this movement could continue spread of the disease, as well replacement of animals for breeding could all be attributed to the high prevalence (Ibrahim *et al.*, 2006; Seleem *et al.*, 2010).

The sex specific prevalence of *Brucella* species infection in sheep revealed that rams had a prevalence of 16.5% while ewes had 4.5%. The infection was seen to have a statistically significant association with sex (p= 0.050) and rams are more likely to be affected than ewes. This finding could be seen as reported by WHO (2006) that *B. ovis* has a great affinity for the reproductive tract of the males than the females. Although the species of *Brucella* detected in this study were not identified, the tropism of the bacteria for the reproductive tract especially in male animals where fructose and erythritol are present in abundance. Erythritol and fructose are

produced due to the catalyst activity of aldose reductase (AR) in the pentose phosphate pathway (PPP) and glycerol has been described either free or as gylceryl-phosphoryl-choline in rams and bulls which is taken by spermatozoa and metabolized through an oxidative process into lactate, whereas, fructose is the major sugar rather than glucose in epididymis, seminal fluid and oviducts of many mammals as it serves as primary energy for spermatogenesis in males (Letesson *et al*., 2017) further support the findings of this study.

The breed specific prevalence of *Brucella* species infection in sheep revealed that Ouda had the highest prevalence of 15.18% while Balami had 11.11% and Yankasa had 0%. More so, there was no statistically significant association between the infection and the breed of sheep. This finding showed that breed is not a risk factor for *Brucella* infection as the pathogen infects all breeds of sheep without preference. This is further described by Tijjani *et al.* (2009) and Junaidu *et al.* (2010) who reported similar findings of the disease in different breeds of sheep showing there is no statistically significant association between seropositivity and breeds of sheep, this indicates that all breeds are susceptible to infection with *Brucella* species.

The finding in this study showed that sex of goats is not risk factor for *Brucella* infection as the pathogen infects both sexes. The breed specific prevalence of *Brucella* species infection in goats revealed that Sahelian had highest prevalence of 50% the West African Dwarf 33.3% and Red Sokoto Goat had 9.28%. Therefore, there was no statistically significant association between the infection and the breeds of goats. This finding also showed that breed of goats is not a risk factor for *Brucella* infection as the organism infects all breeds without preference. There is scarce information about studies done to determine association between infection and breeds of goat using molecular studies in Nigeria but serological reports are available. This finding is similar to the findings of Ogugua *et al.* (2014) and Junaidu *et al.* (2008) who also reported no significant association between seropositivity and breeds of goat, indicating all breeds are susceptible. In other species, Khamesipour *et al.* (2014) reported prevalence of 11.38% in camel's blood sample in Iran using conventional PCR. Also, a prevalence of 6.3% and 12.7% were reported using conventional PCR and qPCR respectively in wild animals in Tanzania (Rosamystica, 2018). These findings though in different species are similar to our findings indicating brucellosis is endemic and there is need for efficient control and prevention strategies

Conclusion and recommendations

From the findings of this study, it can be concluded that PCR is quite a sensitive diagnostic technique than serology, since same samples used for serology were subjected to PCR and 10.72% prevalence was recorded. In sheep, the results showed that rams are more likely to get infected with *Brucella* than ewes because of genital tropism of the organism to the reproductive tract of males due to presence of erythritol and fructose. Even though, molecular techniques are costly which a limitation is, they are highly effective and reliable with less risk to personnel for diagnosis of brucellosis. Further studies should be done up to specie level using conventional PCR or qPCR, also serum samples from veterinarians and abattoir workers should be taken and examined. Abattoir workers should be enlightened about use of personal protective equipment and good practice in the abattoir.

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