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Detection of Bluetongue Virus Nucleic Acid in Ruminants and Camels (*Camelus dromedarius*) in Maiduguri, Nigeria

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Abstract:
***Background*:** *Bluetongue is an infectious, non-contagious, arthropod-borne viral hemorrhagic disease of ruminants and camelids. The disease is caused by the Bluetongue virus (BTV), and the virus infects most ruminants and camelid species but clinical disease is usually only seen erratically in certain breeds of sheep and some species of wild ruminants. The clinical signs observed, include facial edema, hemorrhages, and ulceration on the oral mucosa and coronitis. Bluetongue virus (BTV) outbreaks can cause substantial economic losses, even subclinical infection may carry significant associated costs, including direct loss such as weight loss, meat efficiency, reduced milk yield, infertility, abortion, and death, the indirect loss was largely due to export restrictions for live animals, their semen and some products such as fetal bovine serum. However, the epidemiological pattern and ecology of the Bluetongue virus remain incomplete understood in this part of the country, thus this study was carried out to determine the presence of detectable BTV nucleic acids in the sera of domestic ruminants (sheep, goat, and cattle) and camel (Camelus dromedarius) populations in Maiduguri, Nigeria in order to ascertain the present status of the disease and as well improve the understanding of the epidemiological situation of BTV in the study area*.
***Materials and Methods*:** *A total of 420 blood samples was collected at the point of slaughter from apparently healthy ruminants and camels at the Maiduguri Municipal abattoir and serum were harvested from the blood samples to analyzed for the presence of BTV nucleic acid using the real-time RT-PCR technique*.
***Results**: Out of the 420 sera analyzed for BTV nucleic acid using rtRT-PCR, 17(4.0%) were positive. The prevalence of detectable BTV nucleic acids in the sera of the different species of animals tested was higher in sheep (8.1%), followed in decreasing order of prevalence by goats (3.8%), cattle (1.0%), and camels (0.0%). There was a significant difference ($p<0.0091$) in the prevalence of BTV nucleic acid among the animal species studied. There was no significant association between the distribution of BTV nucleic acid and various age groups ($p=0.0629$) and sex ($p=0.1223$) of the animals. Only the cattle breeds showed a significant ($p=0.0014$) association between the breed and BTV infection.*
***Conclusion:** This study has revealed the presence of Bluetongue virus at a subclinical level in sera of apparently healthy animals in the study area, thus there is a need for a comprehensive nationwide study that cut across a wide range of susceptible domestic and wild animals is recommended to determine the epidemiology of the disease and the circulating serotypes of BTV in Nigeria*.

***Key Word:** Bluetongue Virus, Ruminants, Camels, Real-time reverse transcriptase-polymerase chain reaction, Maiduguri, Nigeria.*

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I. Introduction

Bluetongue is an infectious, non-contagious, arthropod-borne viral hemorrhagic disease of ruminants and camelids transmitted by hematophagous arthropod vectors principally the Culicoides [1;2]. The disease is enzootic in many tropical and temperate regions, coincident with the distribution of competent insect vectors (culicoides) [1]. It is caused by the *Bluetongue virus* (BTV), a type species in the genus *Orbivirus*, of the family *Reoviridae*, that has a segmented double-stranded RNA genome [3]. About 26 serotypes of the virus have been identified [4]. The virus occurs mostly during periods of high temperature and rainfall and usually disappears with the first frost or severely cold weather. Animals infected with BTV present an inapparent to a fatal outcome depending on the infecting serotype and strain of the virus and the species, breed, and age of the infected animal; older animals are generally more susceptible [5]. The disease is characterized by facial edema, hemorrhages, and ulcerations of the oral mucosa and coronet. It ranges from sub-clinical infections in the majority of cases to severe disease and death among infected sheep, cattle and goats usually exhibit the sub-

clinical form of the disease. However, acute infections may occur when the virus enters a virgin region where it is not normally encountered, as observed during the last BTV-8 outbreak in Europe [6]. In endemic regions, cattle and goats are commonly considered to be amplifying hosts of BTV, due to a prolonged viremia that usually occurs in the absence of clinical signs. The worldwide economic losses due to Bluetongue have not been expressed in exact numbers, but the estimate runs into billions of US dollars per annum [7]. The losses are both direct (death, abortions, weight loss, or reduced milk yield and meat efficiency) and most importantly, indirectly as a result of export restrictions for live animals, their semen, and some products such as fetal bovine serum. The epidemiology of BTV infection is poorly defined in much of the world, including extensive portions of Asia, Africa, and the Middle East [8]. In Nigeria there is a dearth of information on the epidemiological situation of BTV, therefore in order to adequately understand the epidemiological pattern and ecology of the virus in this part of the country, this study was carried out to determine the status of BTV disease by detecting the presences of BTV nucleic acids in sera of apparently healthy ruminants and camels in Maiduguri, Nigeria.

II. Material And Methods

This cross-sectional study was carried out on a randomly selected representative subset species of domestic ruminant's animals that include, (sheep, goat, and cattle) and also on the camel (*Camelus dromedarius*) that were slaughtered at Maiduguri Central Municipal abattoir, in Nigeria.

Study design: Cross-sectional study (transverse study)

Study Location: This study was conducted in Maiduguri, the capital of Borno state, it's located in the north-eastern region of Nigeria, the state has the largest livestock market that comprises a livestock population of; cattle (4,800,000), sheep (9,900,000), goats (15,720,000) and camel(80,200), the study was precisely carried out in Maiduguri central municipal abattoir, the types of animals slaughtered in the abattoir daily and their approximate number are: cattle (85-100), sheep (80-100), goats (90-110) and camel (25-40) [9].

Study Duration: The study was conducted between the period; February –December, 2015

Sample size: Total number of 420 ruminants (Sheep, goat, and cattle) and Camels (*Camelus-dromedarius)*

Sample collection: Blood samples were collected at the point of slaughter from the different animals (Sheep, goats, cattle, and camels) and emptied into appropriately labeled sterile plain vacutainer tubes, and transported on ice packs to the Animal Virus Research Laboratory, University of Maiduguri for serum harvesting. The samples were kept in a slanting position at room temperature to clot. Serum samples were harvested from the clotted blood by centrifuging at 1409 x g for 10 minutes. The harvested sera were poured into cryotubes and stored at -20^{0}C until tested.

Laboratory analysis

The serum samples were subjected to a Real-time reverse transcriptase-polymerase chain reaction (rt-RT-PCR) for detection of bluetongue virus nucleic acid as described by Hofmann *et al.*[10]. The method is capable of detecting all known BTV serotypes and strains currently circulating. The assay targets BTV segment 10 (NS3), the process of detection was carried out using a commercially available kit (Qiagen Hilden Germany). The primer sequence and probe used in this study were as follows: Forward Primer (BTV -IVI- F 5' TGG-AYA-AAG-CRA-TGT-CAA-A-3'), reverse Primer (BTV -IVI-R 5'-ACR-TCA-TCA-CGA-AAC-GCT-TC-3'), Probe (BTV-IVI-P 5' FAM-ARG-CTG-CAT-TCG-CAT-CGT-ACG-C-3' BHQ1). The procedure was carried out based on the manufacturer's instruction. Briefly, the serum samples and other extraction reagents (AVE-buffer, carrier RNA, buffer AW1 & AW2, and buffer AVL) were all equilibrated at room temperature before extraction of the RNA. The RNA was extracted by addition of 560µl of prepared Buffer AVL that contained carrier RNA into a 1.5ml of microcentrifuge tube and then 140µl of serum sample was added to the Buffer AVL-carrier RNA in the microcentrifuge tube which was then mixed by pulse-vortexing for 15seconds. The mixed sample was incubated at room temperature (15–25°C) for 10 min. and eventually, 560µl of ethanol was added to the sample and then mixed by pulse vortexing for 15 seconds After mixing the ethanol and the sample thoroughly, 630µl of the mixture–was added to the QIAamp Mini column(a 2ml collection tube) and then centrifuged at 6000 x *g* (8000 rpm) for 1 min, this is followed by two washing steps using 500ul of buffer AW1 and AW2 respectively, and finally, the QIAamp mini-column was placed into a clean new 1.5 microcentrifuge and the RNA was eluted using buffer AVE (an elution buffer). The extracted RNA was immediately converted into cRNA using RNA synthesizes kits. (Qiagen Hilden Germany). The real-time PCR amplification process was performed on 96-well optical PCR plates in a LightCycler480 real-time thermal cycling system, the detection dyes FAM^{TM} and ROX^{TM} were selected for detection of normalization using CXR as the passive reference dye, Denaturation of the cDNA was achieved at 95^{0}C for 20 seconds, the primer annealing temperature was at 60^{0}C for 35 seconds and the primer extension was carried out at 72^{0}C for 35 seconds. The real-time PCR machine was programmed to end the process, by holding the tubes at 4^{0}C for chilling and ending the reaction process. The cycles were repeated for up to 35 cycles to achieve optimal amplification of desired

products, for detection of BTV nucleic acid, samples were considered positives when the cycle threshold (ct) values are less than forty (≤ 40).

Statistical analysis

All the results were appropriately presented in frequency tables and the data obtained were analyzed using JMP version 11 (SAS Institute, Inc. Cary, NC) software. Chi-square test was used for categorical comparison between the various variables and the statistical significance was determined at 95% confidence interval, p-values less than or equal to 0.05 were considered statistically significant.

III. Result

Out of the 420 sera tested with rt-RT-PCR, 17(4.0%) were found to be positive for BTV nucleic acid. The distribution of the positive samples revealed in descending order of prevalence sheep (8.1%), goats (3.8%), cattle (1.0%), and camels (0.0%). There was a significant association ($p<0.0091$) between the infection with BT-virus (BTV nucleic acid) and the animal species studied (Table 1).

Table no 1: Distribution of Bluetongue Virus Nucleic Acid among Ruminants and Camels in Maiduguri, Nigeria

Species	No. Tested	No. positive (%)	95% Confidence Interval	
			Lower limit	Upper Limit
Camel	70	0(0.0)	0.0000	0.0520
Cattle	98	1(1.0)	0.0018	0.0555
Goat	104	4(3.8)	0.0151	0.0948
Sheep	148	12(8.1)	0.0471	0.1364
Total	420	17(4.0)	0.0254	0.0639

The association between infection with BTV and the different species of ruminants and camel is significant. ($X^2 = 11.56$, df = 3, $P< 0.0091$)

Table 2, shows the sex-distribution of BTV nucleic acid, a total number of 146 males and 274 females were screened for BTV nucleic acid, out of these numbers, females recorded the higher rate with 4.7% while males had 2.7%. Among all the animals been studied, Ewe had the highest rate with 8.8%, followed by Buck 8.1%, Ram 4.5%, Cow (cattle) 1.5% and Doe had the less rate of 1.4% while both the Bull and Cow from camel recorded zero (0.0%) rate.

Table no 2: Sex-Distribution of Bluetongue Virus Nucleic acid in Ruminants and Camels in Maiduguri, Nigeria

Specie	Animal terms	Male		Animal terms	Female	
		No. tested	No. Positive (%)		No. tested	No. Positive (%)
Camel	Bull	55	0(0.0)	Cow	15	0(0.0)
Cattle	Bull	32	0(0.0)	Cow	66	1(1.5)
Goat	Buck	37	3(8.1)	Doe	68	1(1.4)
Sheep	Ram	22	1(4.5)	Ewe	125	11(8.8)
Total		146	4(2.7)		274	13(4.7)

The association between infection with BTV and sex among the various species is not significant. ($x^2 = 5.789$, df = 3, P =0.1223).

Table 3: shows the age distribution of BTV nucleic acid in ruminants and camel (*Camelus dromedarius*), out of the total number of 25 young animals that were examined for BTV nucleic acid, 2 (20%) young sheep (lamb) were found to be positive and for the adult animals, out of the 395 that were examined, 15(3.7%) of them were positive, with sheep recorded the highest rate of 7.2%, followed by goat 4.3% and cattle 1.2%.

Table no 3: Distribution of Bluetongue Virus Nucleic Acid among Different Age-Groups in Ruminants and Camels in Maiduguri, Nigeria.

Species	Young Animals (< 1 year)		Species	Adult animals (≥ 1 year)	
	No. tested	No. Positive (%)		No. tested	No. Positive (%)
Calves (Camel)	0	0(0.0)	Camel	70	0 (0.0)
Calves (Cattle)	3	0(0.0)	Cattle	95	1(1.2)
Kid (Goats)	12	0(0.0)	Goat	92	4(4.3)
Sheep (Lamb)	10	2(20.0)	Sheep	138	10(7.2)
Total	25	2(8.0)	Total	395	15(3.7)

The association between infection with BTV and age of the animals is not significant ($x^2 = 5.532$, df = 2, P = 0.0629).

The breed-wise distribution of BTV nucleic acid in sheep, showed that out of the 148 tested animals, only 12 (8.1%) were found to be positive, with Yankasa breed recorded the highest rate 10.2%, then followed in decreasing order, by Balami breed 9.6%, and Uda breed with 5.2% (Table 4)

Table no 4: Breed-wise Distribution Bluetongue Virus Nucleic Acid in Sheep in Maiduguri,Nigeria.

Breeds of Sheep	No. tested	No. Positive (%)	95% Confidence Interval	
			Lower limit	Upper limit
Uda	57	3(5.2)	0.0180	0.1437
Balami	52	5(9.6)	0.0418	0.2062
Yankasa	39	4(10.2)	0.0406	0.2358
Total	148	12(8.1)	0.0470	0.1364

The association between BTV infection and the various breeds of sheep is not significant
($x^2 = 0.891$, df = 2, P = 0.640)

Table 5, shows the distribution of Bluetongue virus nucleic acid in different breeds of goat, where a total number of 104 animals were tested but only 4(3.8%) appears to be positive, out of these four (4) positive, three (3) were from Sahel goat that showed the highest rate 6.3%, while the other one positive is from the West African dwarf goats with the least rate of 3.2%. The remaining two breeds of goat, namely; Borno white and Sokoto red were found to be negative for BTV-nucleic acid.

Table no 5: Breed-wise Distribution of Bluetongue Virus Nucleic acid in Goats in Maiduguri, Nigeria.

Breeds of Goat	No. tested	No. Positive (%)	95% Confidence Interval	
			Lower limit	Upper limit
Sokoto Red	4	0(0.0)	0.0000	0.4899
Borno white	21	0(0.0)	0.0000	0.1546
West African dwarf	31	1(3.2)	0.0057	0.1620
Sahel goat	48	3(6.3)	0.0215	0.1684
Total	104	4(3.8)	0.0151	0.1684

The association between infection with BTV and the various breeds of goats is not significant
($x^2 = 3.622$, df = 3, P = 0.305)

Table 6, Records the distribution of BTV nucleic acid among two breeds of camels, a total of 70 camels were tested for BTV nucleic, out of which 26 of the camels were manga breed and 44 of them were kabashi breeds, however, both of the two tested breed were found to be negative 0(0.0%) against BTV nucleic acid.

Table no 6: Breed Distribution of Bluetongue Virus Nucleic acid in Camels in Maiduguri, Nigeria.

Breeds of Camel	No. Tested	No. positive (%)	95% Confidence Interval	
			Lower Limit	Upper Limit
Manga	26	0(0.0)	0.0000	0.1287
Kabashi	44	0(0.0)	0.0000	0.0803
Total	70	0(0.0)	0.1890	0.2843

The association between BTV infection and the studied breeds of camels is not significant:

Table 7, shows the breed distribution of Bluetongue virus (BTV) nucleic acid among five (5) different breeds of cattle. A total of number of ninety-eight (98) cattle were screened for BTV nucleic acid, out of these total number, ten (10) cattle were Wadara breeds, fourteen (14) White Fulani, eighteen (18) Sokoto gudali, twenty-four (24) Kuri, and thirty-two (32) were the Red-Bororo breed of cattle. Among all the five (5) breeds that were tested for BTV-nucleic acid, only the Red-Bororo breed was found to be positive for BTV nucleic acid with a rate of 1(1.0%)

Table no 7: Breed Distribution of Bluetongue Virus Nucleic acid Amongst various breeds of cattle in Maiduguri, Nigeria.

Breeds of Cattle	No. Tested	No. Positive (%)	95% Confidence Interval	
			Lower Limit	Upper Limit
Wadara	10	0(0.0)	0.0000	0.2775
White Fulani	14	0(0.0)	0.0000	0.2153
Sokoto Gudali	18	0(0.0)	0.0000	0.1759
Kuri	24	0(0.0)	0.0000	0.1380
Red-Bororo	32	1(3.1)	0.0056	0.1575
Total	98	1(1.0)	0.0018	0.0555

The association between infection with BTV and the various breeds of cattle is significant.
($x^2 = 1.240$, df = 4, P = 0.0014).

IV. Discussion

Bluetongue virus is an infectious, non-contagious, arthropod-borne viral disease of ruminants, that is categorized and listed among the transboundary animal disease, the disease can rapidly spread across many countries borders [11]. Therefore, the use of gene-specific primers in direct amplification of a viral nucleic acid has modified bluetongue virus diagnosis and as well detection method using PCR has been one of the recommended tests by OIE for international trade of animal products. In this present study detection of BTV nucleic acid in the sera of ruminants and camels (*Camelus dromedarius*) was conducted using rt-RT-PCR, the result of the study showed an overall rate of 4.0%. This is an indication that there is a sporadic infection of these animals with the Bluetongue virus (BTV) and unfortunately, vaccination against BT is not done in the study area, hence the detected nucleic acids could have been as a result of natural infection of the animals with BT-virus. The overall detected rate of BTV nucleic acid that was recorded in this study is lower than the 88.9% observed by Toye *et al.* [12] among calves in Kenya and 18.7% observed among clinically suspected sheep in Iran [13]. These differences could be probably due to the differences in the geographical locations of the study sites or as a result of the health status of the animals at the time of sampling and as well the period of the year when the animals were sampled.

Findings based on species distribution of BTV-nucleic acid in this study revealed that sheep had the highest rate with 8.1% while the least rate was observed in cattle with 1.0%, this agrees with the previous study conducted by Anjaneya *et al.* [14], who reported a higher rate in sheep 30% and a lower rate in cattle 27.1%, it's also in-line with the study conducted by shad *et al*. [16], who detected BTV antigens in eight sheep that were subcutaneously inoculated with BTV serotype-10 using RT-PCR, but it disagrees with the study carry-out by Polci *et al*. [15], who reported positive BTV RNA in 64 sentinel cows that are seroconverted to BTV serotype 2 and 16 using real time PCR in Italy, The higher rate that was observed in sheep in this study could have been probably due to the fact that the sheep are more permissive and susceptible to BT-virus as compared to other species and also they exhibit their potential role as a major subclinical carriers of BT-virus due to development of acquired immunity, which occurs as a result of continuous exposure to the virus or it could be as a result of their involvement in the micro-epidemiology of the disease.

The sex-wise distribution of BTV nucleic acid of the subjects under study shows that generally, females recorded the overall highest rate with 4.7% while the male had 2.7%, and amongst all the female's species that were screened, the Ewe (sheep) had shown the highest rate of 8.8% and cow (female cattle) got the least rate 1.5%, while for their male counterparts, the highest rate was observed in buck (male-goat) 8.1% and the least rate in ram (male-sheep) 4.5%. The highest rate that was observed in females is in agreement with previous study conducted by Bitew *et al*. [17], who also reported a higher rate in sheep(female) with 34.3% and least rate in ram(male) 25.0%, and also it further supported the study conducted by Mayo [18], who reported a higher rate of BTV-viral RNA in adult ewe flock 7.41% than the adult ram flock 5.56%, but despite the differences in the rate of the disease among the two sexes, bluetongue virus by its nature has no specific preference to any sex, both male and female animals have equal chances of acquiring BTV infection, this was observed in a study conducted by Rajkhowa *et al*. [19], in India, were they revealed an equal susceptibility of both males and females to BTV infection.

In terms of age-distribution of BTV infection, between the two age groups (young and adult) that were screened for BTV-nucleic acid, young animals (≤ 1year) recorded the highest rate, with over 20% rate in lamb (young) as against 7.2% rate in adult sheep, this is in agreement with the previous study conducted by Mayo [18], who also documented a higher rate of 17.59% in lambs and 7.41% in adult sheep in Colorado State University, USA and also it's in support with findings by Sreenivasulu *et al*. [20], who revealed that sheep that were aged 6-12month were more susceptible than the adult's sheep, but it's contrary to report by Shringi *et al*. [21], who point-out that BTV-infection is common among adult animals than young animals. The higher rate of BTV viral nucleic acid that was observed in lambs in this study could be probably due to their naïve immunological status and first exposure to BTV, on the other hand, the presence of BT-virus in adult animals could be probably attributed to the continuous roaming of the animals in search of fodder and movement of adult stock for marketing.

Moreover, among all the breeds of animals that were screened for BTV nucleic acid, only the Red-Bororo breed of cattle tested positive and recorded 3.1%, this finding is contrary to a previous study conducted by Khaled *et al*. [22], who reported a zero (0.0%) rate of BTV-RNA in native breeds of sheep and goats screened for the virus in Egypt, but Worwa *et al.* [23], have reported a remarkably high virulence of BT-virus in indigenous breeds of sheep in Switzerland, besides, Coetzee *et al*. [24], has also stated that there is breed predisposition in the occurrence of bluetongue disease in sheep. The presence of BTV-nucleic acid that was observed in the Red-Bororo breed of cattle could be probably due to some certain genetic disorders or genetic make-up that makes such breeds to be prone to BTV-infection, whereas the remaining negatively tested breeds could probably harbor some certain resistance genes that makes them to be less predisposed to the infection [25].

V. Conclusion

In conclusion, Bluetongue virus-nucleic acid was found to be present at a subclinical level in sera of apparently healthy animals in the study area, the result revealed that sheep had the highest rate, which implies that they are the most sub-clinically affected species. Specific measures indicate that young age and certain breed predispose such animals to BTV-infection, however, despite the fact the high rate of infection was observed in female animals, bluetongue virus by its nature has no preference to sex, it can equally affect both male and female animals at the same rate. Thus it is recommended that screening tests for the virus should be conducted in the field and thereafter other complex virological tests such as the multiplex PCR in line with viral isolation in cell cultures or embryonated chicken egg should be carried out in other to ascertain another emergence of new BTV serotypes and the current status of the virus in the affected areas and the country at large, more so, a longer period survey, should be conducted which will include entomological and virological surveillance that will ascertain the actual seasonality or period of BTV transmission, thus it will provide additional information that will be implemented as control strategies of the disease in the affected areas.

Conflict of Interest

The authors of this work declared that there was no conflict of interest. None of the authors have a financial or personal conflict of interest related to this study.

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