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DNA BARCODING OF IXODID TICKS INFESTING CATTLE AND SHEEP IN NSUKKA, NIGERIA

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ABSTRACT

Ticks constitute one of the major health challenges to livestock production in Nigeria. They are particularly important due to their blood-feeding activity causing anaemia and as vectors of pathogens causing many livestock diseases. Accurate identification of ticks infesting livestock is fundamental for effective control of tick and tick-borne diseases. We integrated morphological identification and DNA barcoding to unambiguously identify the species of ticks and assessed their prevalence on cattle and sheep in Nsukka, Nigeria. All the animals in the farm: 37 cattle and 22 sheep were examined. All the cattle examined (100%) and 9 (40.09%) of the sheep were infested with ticks. The ticks *Amblyomma variegatum* and *Rhipicephalus microplus* were found on cattle and sheep in Nsukka. A total of 317 ticks were collected out of which 272 were on cattle and 45 on sheep. Of the total number of ticks collected, 152 (47.95%) were *A. variegatum* while 165 (52.05%) were *R. microplus.* Tick infestation was significantly higher on cattle than sheep ($p<0.05$, $\chi^2 = 3.902$, $p = 0.0482$, $n=59$). The barcode region of COI gene was successfully amplified and sequenced in all the samples of ticks analysed. Samples of *A. variegatum* were identified at 99.70-100%. Similarly, samples of *R. microplus* were identified at 100%. Phylogenetic analysis clustered *A. variegatum* and *R. microplus* with their respective species from GenBank on phylogenetic tree. The ticks exhibited morphological ambiguity and sexual dimorphism, but DNA barcoding resolved the identities of the ticks. Integrative taxonomy is the best for tick identification.

Keywords: COI gene, identification, livestock, prevalence, taxonomy

INTRODUCTION

Ticks are blood-sucking ectoparasitic arachnids of the Order Acari and sub-order Ixodida (Walker *et al*., 2014). In the course of their blood sucking habits, ticks have been implicated as vectors of pathogens such as *Anaplasma, Rickettsia, Theileria, Babesia,* and *Borrelia* species. In addition, ticks cause anaemia, irritation, damage to hides and skin, hair loss and reduction in livestock production (Branscheid and Schroer, 1997; Jongejan and Uilenberg, 2004; Shemshad *et al*., 2011; Nnabuife *et al*., 2021). Globally, an estimate of annual loss of 22-30 billion US$ in livestock production due to ticks has been reported (Lew-Tabor and Valle, 2015). Ticks in the genera *Amblyomma*, *Hyalomma* and *Rhipicephalus* constitute the greatest health challenges to livestock (Rajput *et al*., 2006). In Africa, about 50 tick species are of veterinary importance (Walker *et al*., 2014).

Accurate identification of ticks infesting livestock is crucial in management of tick infestation and control of tick-borne diseases (Lv *et al*., 2014). Tick identification has been primarily by morphological identification of the adult stage (Apanaskevich and Horak, 2008; Apanaskevich *et al*., 2008; Dantas-Torres *et al*., 2013). However, morphological identification has so many limitations such as not being able to identify the developmental stages, similarities among species, lack of taxonomic keys for some species, is tedious and need special training, and in the case of ticks, those engorged with blood present problems in identification (Caporale *et al*., 1995; Guglielmone *et al*., 2006). One of the major cattle ticks *Rhipicephalus (Boophilus) microplus* is a species complex among which are species that have many morphological similarities and thus are very difficult to identify morphologically (Roy *et al*., 2018).

Many works have been done on the ticks infesting cattle in different parts of Nigeria (James-Rugu and Iwuala, 2002; Ikpeze *et al*., 2011; Obadiah and Shekaro, 2012; Lorusso *et al*., 2013; Oduguwa *et al*., 2013; Eyo *et al*., 2014; Musa *et al*., 2014; Aminu, 2015; Opara and Ezeh, 2016; Kamani *et al*., 2017; Adane *et al*., 2019). Of all the works that involved identification of cattle ticks in Nigeria, only Kamani *et al*. (2017) employed molecular method using the nuclear ITS2 gene in identifying *R. microplus*. Molecular methods accurately capture the differences among species (Bezeng *et al*., 2017) and complement morphological data in species identification. Molecular sequence data involving mtDNA COI gene has stronger patterns of genetic variability than those of nuclear regions (Li *et al*., 2014a), and the COI barcode region has been a verified effective and informative tool employed in animal identification (Hebert *et al*., 2010; Pawlas-Opiela *et al*., 2010; Li *et al*., 2014b) including ticks (Lv *et al*., 2014; Zhang and Zhang, 2014; Csordas *et al*., 2016; Ghosh *et al*., 2020; Davari *et al*., 2021). Integrative morphological and molecular identification of ticks gives a clear and more reliable result compared to single identification method (Ghosh *et al*., 2020). DNA barcoding using COI gene has been recommended as the gene of choice for ticks when compared to nuclear genes (Lv *et al*., 2014). Hence, the objectives of this study were to employ DNA barcoding of mtDNA COI gene to complement morphological method to identify ixodid ticks and access their prevalence in livestock of Animal Farm, University of Nigeria, Nsukka (UNN).

MATERIALS AND METHODS

Study area

The study was conducted in the Animal Farm, Department of Animal Sciences, University of Nigeria, Nsukka. The Animal Farm is located inside UNN which lies between Latitudes 6°51′0″ N, 6°52′30″ N and Longitudes 7°24′0″ E, 7′26′30″ E (Figure 1). The natural day length for Nsukka is 12–13 hours and the average annual maximum and minimum temperatures are 29.7˚C and 21.0˚C respectively. The relative humidity ranges from 34 to 78% (Inyang, 1978). Nsukka is a derived

savanna zone with vegetations consisting mainly of grasses with sparse distribution of tall trees. The farm is fenced and does not allow unauthorized access. However, the cattle and sheep are taken outside occasionally for grazing.

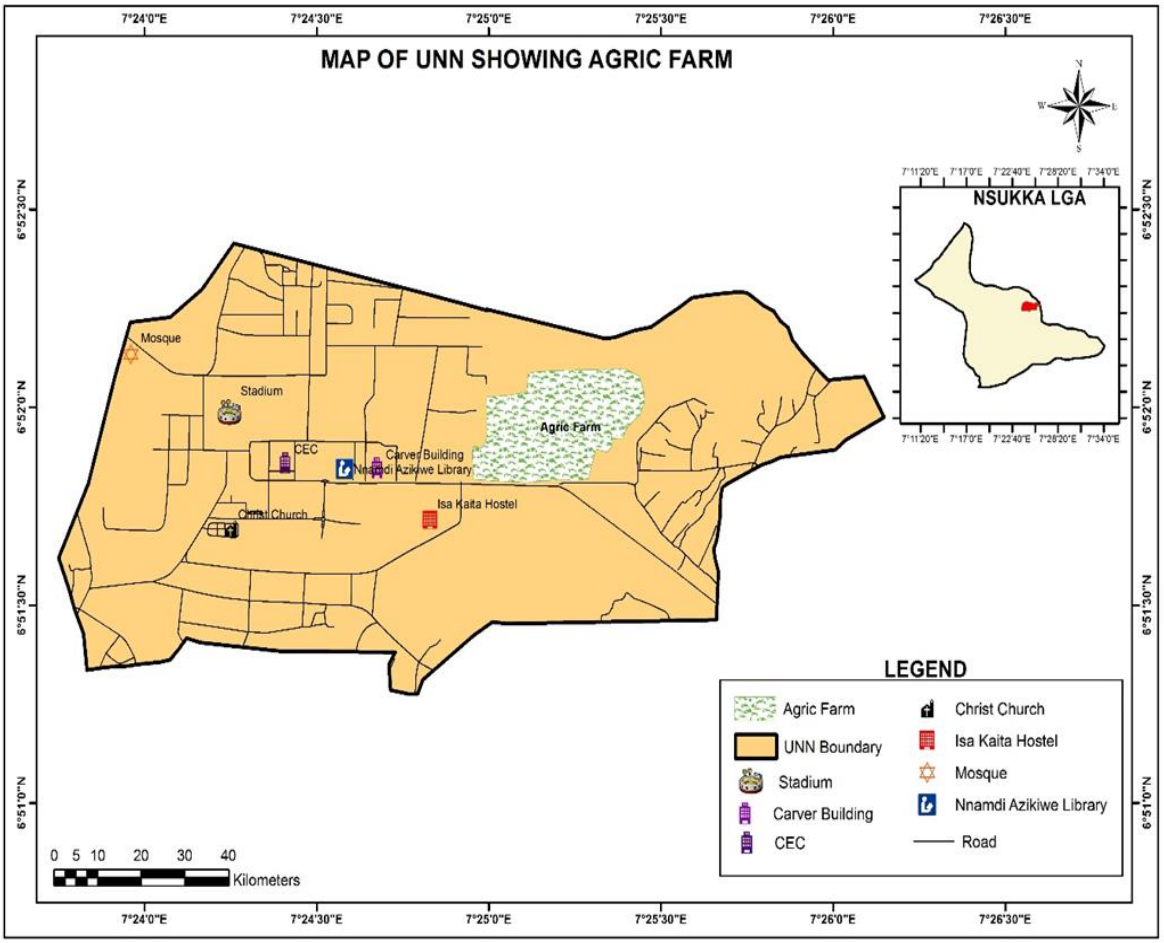

Figure 1: Map of the study area

Collection of Ticks

A total of 37 cattle and 22 sheep are in the farm, and all were examined. Two breeds of cattle are in the farm, the *Muturu* and *Ndama*. The source of the cattle in the southern Nigeria including the study area is Kano State (Bello, 2020). Ticks were collected from cattle and sheep in the Animal Farm by gently pulling them from the site of attachment to the host using the hand or forceps. The ticks collected were grouped according to their morphological similarities and preserved for further studies. Tick samples for morphological identification were preserved in 70% ethanol while samples for DNA barcoding were preserved in absolute ethanol. Ticks were collected from the animals for three months (June – August 2021). The species and numbers of ticks collected were recorded for the sexes of cattle and sheep, and breeds for cattle.

Identification of the ticks collected

The ticks collected were taken to the Entomology Laboratory, Department of Zoology and Environmental Biology, University of Nigeria Nsukka where they were compared with voucher specimens in the lab. Over 1000 samples of ticks collected from cattle in Nsukka are in the Entomology laboratory as voucher specimens. The voucher specimens were re-examined in addition to the samples collected in this study. The morphological characters of the ticks were further examined thoroughly with the aid of Stereo microscope and identified to species level using keys by Walker *et al*. (2014). Four different morphological forms of the ticks were identified which are the same for both the museum vouchers and the samples collected for this study. The ticks according to the voucher specimens are four different species of ticks however, Walker *et al*. (2014) identified them as male and female of two different species of ticks. Two samples of each morphological form from the voucher samples in the lab and the samples collected for this study were selected for DNA barcoding. In total, 16 ticks were selected for DNA barcoding for this study. Thereafter, the different morphological forms of the ticks identified were transported to the African Centre for DNA Barcoding, Department of Botany, University of Johannesburg, South Africa for DNA barcoding.

DNA Barcoding of the Ticks

Genomic DNA was extracted from the different morphological forms of the ticks using Zymo Research Quick-DNA™ Miniprep Plus Kit. DNA products were viewed on a 1.5% agarose gel to confirm the success of the DNA extraction. Thereafter, the barcode region of the COI gene of the extracts from the ticks were amplified using the primers LCO1490 5′ GGTCAACAAATCATAAAGATATTGG 3′ and HCO2198 5′ TAAACTTCAGGGTGACCAAAAAATCA 3′ (Folmer *et al*., 1994). All reactions were performed in a total volume of 25µl per sample. The master mix was made up of 12.5µl Taq DNA Polymerase, 2x Master Mix Red-Ampliqon, 0.3µl of each primer and 0.5µl Magnesium chloride. Additionally,

0.8µl of bovine serum albumin (BSA) (3.2%) was added to all PCR reactions, as well as 0.5µl of dimethyl sulphoxide (DMSO) (4.5%). Finally, 1µl of DNA was added to the tube. PCR amplification was performed in an Applied Biosystems Proflex™ PCR System (Thermo Fisher Scientific) using the following programmes: pre-melt at 94°C for 3 min, denaturation for 1 min at 94°C, annealing at 48°C for 1 min, extension at 72°C for 3 min (for 28 cycles), followed by a final extension at 72°C for 7 min. Prior to cycle sequencing, the PCR products were visualized on a 1.5% agarose gel and subsequently purified using ExoSAP-ITTM (GRiSP, Lda, Portugal) reagent protocol (1.8 µl H2O, 0.20 µl Exo1, 0.40 µl FastAP) – incubation at 37˚C for 30 min followed by 80˚C for 15 min.

Cycle sequencing reactions were carried out using BIGDye TMv.3.2 Terminator Kit (Thermo Fisher Scientific, Massachusetts, USA). The purified PCR amplicon were sequenced in both forward and reverse directions using their respective forward and reverse primer. The following conditions were used: 26 cycles for 10 seconds denaturation at 96°C, 5 seconds annealing at 50°C and 4 minutes extension at 60°C. Cycle sequencing products were precipitated in ethanol and sodium acetate to remove excess dye terminators before sequencing on an ABI 3130xl genetic analyser.

Data Analysis

Data was analysed using Statistical Packages for Social Sciences version 23.0 (IBM Corporation, Armonk, USA). Prevalence of ectoparasite infestation was compared using chi-square analysis. Level of significance was set a $p < 0.05$. Parasite prevalence and intensity were calculated using the formulae:

$$\text{Prevalence (\%)} = \frac{\text{Number of infected host}}{\text{Number of examined host}} \text{ x } \frac{100}{1}$$

$$\text{Mean intensity} = \frac{\text{Total number of parasites}}{\text{Number of positive individuals}}$$

Sequence Data Analysis

Raw sequence data was BLAST Search in GenBank, and the sequences with the highest percentage score and query cover were downloaded and used to align the sequences. The sample sequences were aligned with *Amblyomma variegatum* Fabricius (Accession Numbers: GU062743 and KU568507) and *Rhipicephalus microplus* Canestrini (Accession Numbers: MN294738 and MT249801) in MEGA7 (Kumar *et al*., 2016). The aligned sequences were edited in Microsoft Word by removing the primer sequences at the extremes and crosschecking the Genetic Analyzer chromatograms where the sequences did not match and replacing the appropriate bases. The sequences were deposited in GenBank under the accession numbers (LC731916, LC731917 – LC731930, LC731931).

Phylogenetic Analysis based on COI Gene

The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). This was after model testing revealed that Tamura 3 – parameter model and Gamma distribution are the best fit model for the evolutionary analysis. The tree with the highest log likelihood (-1507.70) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites [2 categories (+G, parameter = 0.6002)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 33 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 539 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016)

RESULTS

Identification of the Ticks Infesting Cattle and Sheep in the Animal Farm

Ticks identified in the farm are *Amblyomma variegatum* and *Rhipicephalus microplus.* The ticks exhibited varying morphological features. The engorged ticks were more than 10 times the size of unengorged ticks. The unengorged *A. variegatum* has prominent white markings on the limbs and are typically black in colour for females but the males have variegated brownish dorsal region. The female of *A. variegatum* is devoid of the variegated markings seen on the dorsal region of the male. The legs of *A. variegatum* has pale rings and scutum, and the mouthparts are long. *Rhipicephalus microplus* have short mouthparts. The engorged tick is brown with shot legs. The legs are slender with no pale rings. These variations in the morphology of the ticks complicated the use of morphology in the identification of the various morphological forms collected. Four different morphological forms of the ticks were identified which are the same for both the museum vouchers and the samples collected for this study. The four morphological forms are male and female of *A. variegatum* and *R. microplus*. The male of *A. variegatum* was correctly identified in the museum voucher while the female was deposited as *A. maculatum*. The engorged female of *R. microplus* was correctly identified in the museum voucher while the male was deposited as *R. annulatus*.

The primer pair LCO1490/HCO2198 successfully amplified and sequenced the barcode region of COI gene in all the samples analysed. Samples of *A. variegatum* showed 99.70-100% similarity with *A. variegatum* from GenBank. Similarly, samples of *R. microplus* identified *R microplus* in GenBank at 100% similarity. Phylogenetic analysis based on 658 bp COI gene clustered all *A. variegatum* and *R. microplus* with their respective species from GenBank on a phylogenetic tree. The phylogenetic tree has two clades, one for *A. variegatum* and the other for *R. microplus* (Figure 2). DNA barcoding and phylogenetic analysis for the various morphological forms resolved the identity of the different tick species.

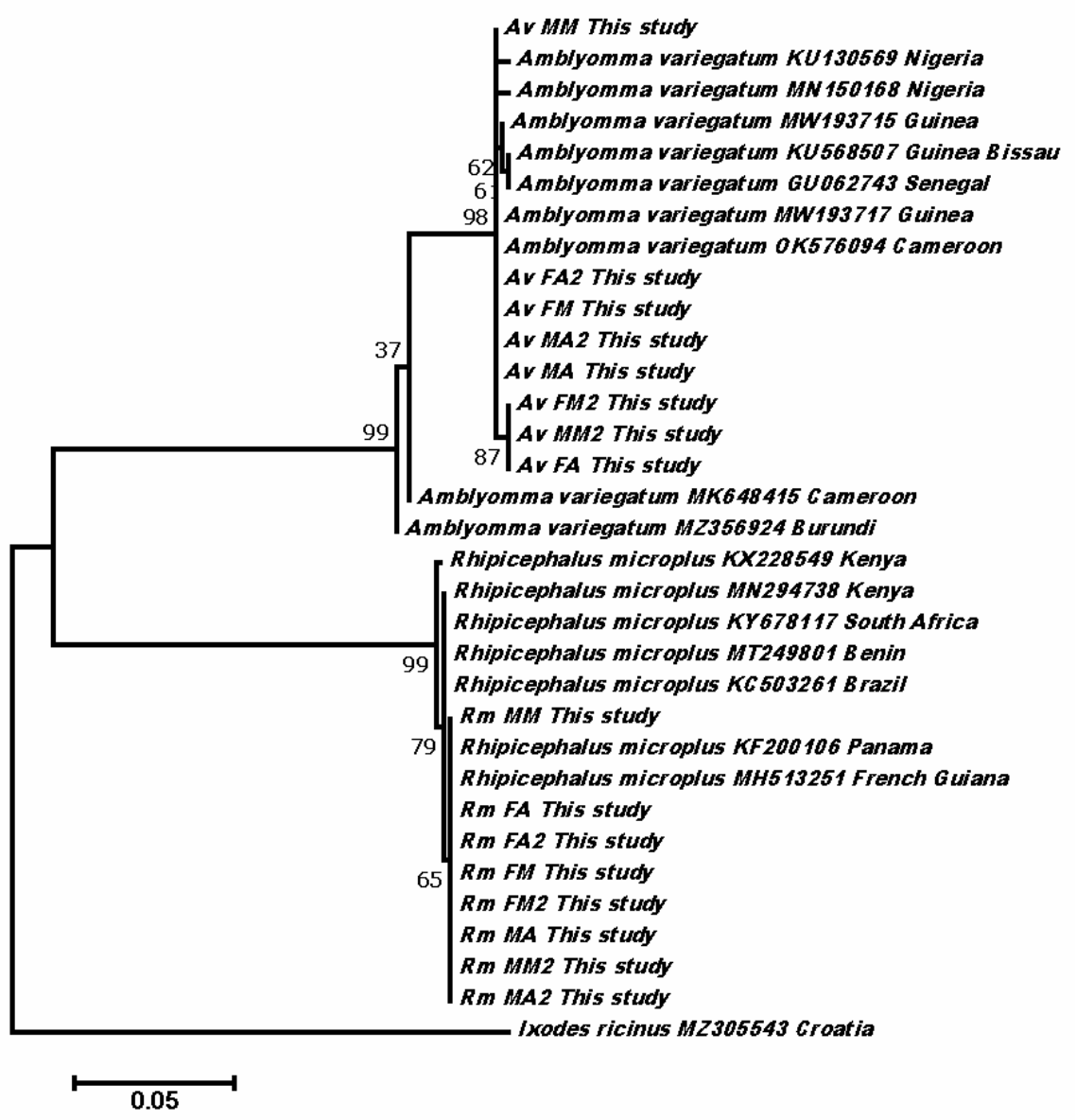

Figure 2: Phylogenetic tree of the ticks based on COI gene

Prevalence and Intensity of Ticks Infesting Cattle and Sheep in the Animal Farm

The two species of ticks identified infested both sexes and breeds of cattle and sheep in the farm. A total of 317 ticks were collected from the farm, 272 on cattle and 45 on sheep. Of the total number of ticks collected, 152 (47.95%) were *A. variegatum* while 165 (52.05%) were *R. microplus*. All the 37 (100%) cattle examined had ticks while only 9 (40.91%) of the 22 sheep examined had ticks. The prevalence of tick infestation was significantly higher on cattle than sheep ($p<0.05$, $\chi^2 = 3.902$, $p = 0.0482$, $n=59$). The numbers and mean intensities of *R. microplus* infesting cattle are higher than *A. variegatum*. However, for the sheep, the numbers and mean intensities of *A. variegatum* was higher than *R. microplus* (Table 1). The mean intensity of tick infestation was higher in the two breeds of female cattle than the males. On the other hand, the mean intensity of tick infestation was higher in males than the females in sheep. However, there was no significant difference in the tick infestations between male and female of the farm animals examined. The two breeds of cattle did not show significant difference in tick infestation (Table 2).

Table 1: Ticks infesting cattle and sheep in Animal Farm UNN

	Parasite species	Number infected (%)	Number recovered	Mean intensity
Cattle	*Ambylomma variegatum*	37 (100)	129	3.49 ± 2.0
	Boophilus microplus	37 (100)	143	3.86 ± 3.0
Sheep	*Ambylomma variegatum*	9 (40.91)	23	2.56 ± 2.0
	Boophilus microplus	9 (40.91)	22	2.4 4± 3.0
		$\chi^2 = 3.902$, p = 0.0482		

Table 2: Prevalence of ectoparasite infestation in relation to sex of cattle and sheep in Animal Farm, UNN

		Number of animals examined	No infected (%)	No. of ticks collected	Mean intensity
Cattle Breed	Sex				
Ndama	Male	10	10 (100)	69	5.3 ± 2.0
	Female	8	8(100)	90	7.8 ± 3.1
Muturu	Male	9	9 (100)	50	2.9 ± 1.0
	Female	10	10 (100)	63	3.5 ± 3.2
			χ^2 = 0.00, p = 1.00		
Sheep	Male	10	5 (50.0)	23	2.1 ± 1.3
	Female	12	4 (33.3)	22	2.0 ± 1.0
			χ^2 = 0.261, p = 0.6095		

DISCUSSIONS

The ixodid ticks infesting cattle and sheep in the animal farm are *A. variegatum* and *R. microplus*. The morphological identification was complemented by DNA barcoding to arrive at an undisputable identity of the ticks. The different morphological forms identified and deposited in the Entomology Laboratory as four different species were identified by Walker *et al*. (2014) as two different species. Following description by Walker *et al*. (2014) the samples are *A. variegatum* (male and female) and *R. microplus* (male and female). The identities of the ticks according to Walker *et al*. (2014) were confirmed by DNA barcoding. Thus, morphological identification could be misleading especially when expert taxonomists were not involved in the process.

Rhipicephalus microplus was more prevalent in the farm and constituted 52.05% of the total ticks collected while *A. variegatum* accounted for 47.95% of the total ticks recovered. The findings in this study corroborated the results of earlier investigators in different parts of Nigeria. The result of this study agrees with Daodu *et al*. (2021) who recorded *R. microplus* and *A. variegatum* infesting cattle in Nigeria. Daodu *et al*. (2021) similarly recorded higher number of *R. microplus* than *A. variegatum* on cattle in Nigeria. In Edo State Nigeria, Adane *et al*. (2019) recorded *A. variegatum* and *R. micropolus* on cattle but in addition to *Rhipicephalus* sp. In line with our result, Adane *et al*. (2019) similarly recorded higher prevalence of *R. microplus* than *A. variegatum* on cattle. However, the parasite intensity of 3.49 ± 2.0 and 3.86 ± 3.0 for *R. microplus* and *A. variegatum* respectively recorded on cattle in this study is lower than 29.83±13.79 and 8.29±3.77 for the same species recorded by Adane *et al*. (2019). The low parasite intensity recorded on cattle in this study could be attributed to the small number of cattle in the farm which limited the number of cattle sampled in addition to the presence of sheep in the farm which also served as alternative host for the ticks. In Dodoru Market, Kebbi State, Abdullahi *et al*. (2018) recorded five species of ticks on cattle but *A. variegatum* was the only one among the five recorded in animal farm in Nsukka. However, in line with this study, Abdullahi *et al*. (2018) found that the genus *Boophilus* has a higher prevalence than *A. variegatum* on cattle. In south-eastern Nigeria, Ikpeze *et al*. (2011) recorded four species of ticks on cattle but *A. variegatum* was the only species reported in their study that was also found in this study. In line with this study, Ikpeze *et al*. (2011) found *A. variegatum* to be the least prevalence tick on cattle. Kamani *et al*. (2017) similarly recorded *A. variegatum* and *R. microplus* but in addition to *Hyalomma* spp. on cattle across Nigeria. However, Kamani *et al*. (2017) found *A. variegatum* to be the highest prevalent tick on cattle while our study found that *R. microplus* was the most prevalent tick on cattle. Lorusso *et al*. (2013) recorded eleven species of ixodid ticks on cattle in central Nigeria. Of the two species recorded in this study, only *A. variegatum* was among the eleven species recorded by Lorusso *et al*. (2013). Nnabuife *et al*. (2021) recorded *A. variegatum* on sheep in Plateau State Nigeria but no *R. microplus*. Due to morphological ambiguities among tick species and the likelihood of misidentifying ticks when only morphological features are used, the species and their prevalence as recorded by earlier investigators across Nigeria might not be a true representative of the actual species and their prevalence.

Of all the study on ticks infesting cattle in Nigeria, only Kamani *et al*. (2017) applied molecular method to identify *R. microplus*. However, Kamani *et al*. (2017) used ITS2 region of gene, but this study employed COI gene in barcoding the tick samples collected. DNA barcoding based on COI gene has been verified as effective and more informative in identification of animal specimens (Hebert *et al*., 2010; Pawlas-Opiela *et al*., 2010; Li *et al*., 2014a). This study proved that morphological identification of ticks cannot be solely relied upon in identification of ticks of farm animals. Earlier study in the area that employed only morphological identification identified male and female samples of *A. variegatum* and *R. microplus* as different species. This is due to the wide sexual dimorphism between the species and the difficulty of using keys and vouchers in identification of tick samples. It is therefore necessary to reassess the tick species infesting farm animals across Nigeria using DNA barcoding of COI gene for appropriate management of the pest burden and surveillances of the pathogens vectored by each species of ticks.

All the cattle examined in this study had both species of ticks, that is, a prevalence of 100%. The 100% prevalence of tick infestation recorded in this study contradicts the findings of Abdullahi *et al*. (2018) and Adane *et al*. (2019) who recorded a lower prevalence of 44.4% and 21.5% respectively on cattle in different parts of Nigeria. The 100% prevalence of tick infestation on the cattle recorded in this study could be because of the small number of cattle in the animal farm. In addition, the cattle are mostly confined in the farm hence, they are always in contact with the same environment and the ticks inhabiting the area. In addition, due to the small number of animals in the farm little attention is paid to routine tick control in the farm.

In this study, the prevalence of ticks was higher among the female cattle than the males. This finding is in line with the result of Adane *et al*. (2019) in Edo State, Nigeria who recorded higher infestation of ticks among the female cattle than the male. This could be that the ticks prefer female cattle than the males. In addition, pregnant female cattle and the milking ones are commonly confined to the farm for extra pastoral care when others are taken out to graze in the field. This habit of keeping the females in one place could be a factor for preference by the ticks.

CONCLUSION

In conclusion, there is the likelihood of misidentifying ticks if only morphological method is employed, even when experts are involved, because of the varied morphological differences among the ticks. This study has proven this and recommends reassessing the ticks of farm animals across Nigeria using DNA barcoding.

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