Suggested Revisions by Reviewer

MOLECULAR CHARACTERIZATION OF *ANAPLASMASPP* ISOLATED FROM CATTLE SLAUGHTERED AT CENTRAL ABATTOIR BAUCHI, BAUCHI STATE, NIGERIA

ABSTRACT

Background: Bovine anaplasmosis is a disease of cattle caused by *Anaplasma*haemoparasite. It has significant effects on the animal's health but has been overshadowed by other haemoparasitic diseases like Trypanosomiasis and Babebiosis. The discovery of new species with zoonotic potentials in ~~the~~ recent years has given it more significance. This study was conducted to molecularly characterize *Anaplasma species* isolated from cattle slaughtered at the Bauchi central abattoir.

Materials and Methods: Blood samples were collected from the severed jugular vein of the animals at the point of slaughter and collected samples were taken to the laboratory for analysis.DNA extraction was carried out using the Quick -DNA™ Miniprep kit catalogue Nos. D3024 & D3025 using the Quick protocol. Extracted DNA from the blood sample was amplified with specific primers targeting the 16S rRNA (≈430 bp) genes in the *Anaplasma* parasite. PCR was performed using BioinGentech Veterinary PCR kits (Concepcion, Chile) and amplified DNA fragments of the 16sRNA gene from the *Anaplasma* spp. isolates were directly sequenced using the Sanger method.

Results: Phylogenetic shows thatthe 16S rRNA nucleotide sequences of *Anaplasmaspecies* obtained from this study clustered into four groups. *Anaplasma species* from Bauchi state clustered with other*Anaplasma* sequences and based on multiple alignment, the sequence presented nucleotide differences between them suggesting that different strains are circulating in the state based on the analyzed data.TheDNA sequencing result of the 16SrRNA also revealed that *Anaplasma platys* and *Anaplasmaphagocytophylum*are circulating among cattle herds in the state and this is of public health implication because *A. phagocytophilum* is zoonotic.

Conclusion:.Five nucleotide sequences were obtained from this study and deposited in Genbank under the following accession numbers: OQ538141 for *Anaplasma platys*, OQ538142 for *Anaplamamarginale*, OQ538143 for *Anaplasmaphagocytophilum* and OQ538144 to OQ538145 for uncultured *Anaplasma species*.

Keywords: Anaplasma, cattle, abattoir,Bauchi, Nigeria

1. INTRODUCTION

*Anaplasmaspecies*is a haemoparasitic organism (protozoan) and is the causative agent of anaplasmosis.Up to 17 different tick vector species (including *Dermacentor*, *Rhipicephalus*, *Ixodes*, *Hyalomma*, and *Argas*) have been reported to transmit *Anaplasmaspp*. [1]. Not all of these are likely significant vectors in the field, and it has been shown that strains of *A.marginale* also coevolve with particular tick strains. *Rhipicephalus spp.* are major vectors in Australia and Africa, and

Dermacentorspp. have been incriminated as the main vectors in the USA[2].According to a study[3],bovine anaplasmosis primarily caused by *A. marginale*, is considered as one of the most prevalent and costly tick-borne diseases of cattle globally[4].It is reported asbeing~~that it is~~ endemic in most of the cattle-farming areas in Africa.

There is a huge economic impact of anaplasmosis from different countries which is corroborated by the widespread occurrence of the *Anaplasmaspp.* in cattle [5]. The disease is ~~continuously~~becoming a serious concern for the animal breeding system, as the infection puts an additional burden on veterinary care by reducing the body weight of animals, decreasing milk production, and frequently causing abortions leading to death [6]. The red blood cells of cattle and wild ruminants are chosen by *A. marginale andA. centrale* as a site of infection, while small ruminants presenting the same cells ~~to be infected~~ are infected~~encountered~~ by *Anaplasmabovis* causing anaplasmosis, targetingsmall mammals and ruminants, which results in the infection of monocytes. According to [7], the prevalence rate is dependent on the type of species infected and the diagnostic method used. A study on molecular characterization conducted in some states by [8], revealed the presence of mixed haplotypes in both *A. platys* and *A. marginale*in cattle in Nigeria.

Traditionally, the identification, description and classification of *Anaplasma species* are based on the morphological characteristics using microscopic examination of Giemsa-stained thin or thick blood smears with or without serology. However, recent studies have demonstrated that *Anaplasma* and related piroplasmids are not monophyletic and can co-infect hosts; therefore, they require the use of molecular tools for the identification and characterization of these cryptic and polyphyletic species [9][10].It is reported that the small subunit ribosomal RNA (16S rRNA) gene is widely used for detection and phylogenetic analysis of protozoan organisms due to its high level of conservation.

There is a need for the accurate identification of the causative agents of anaplasmosis considering the widespread distribution and the huge economic losses associated with the disease in order to design and facilitate implementation of effective control measures.

Molecular techniques have become very useful tools in parasite identification since ~~it~~theytarget~~s~~ the genes of the organism thus giving ~~a~~ more accurate and definite information.

The detection and characterization of *Anaplasmaspp.* are performed using molecular tools in which sensitive and specific nucleic-acid based techniques targeting several genes such as the 16S *rRNA*, heat-shock protein (*groEL*), major surface protein-1a (*Msp1a*), *Msp2, Msp4* and *Msp5* have been developed to detect and characterize *Anaplasmaspp.* infection in animals [3].

PCR ~~has been characterized as~~is considered to bethe gold standard diagnostic approach for anaplasmosis [11],but it has not been used preferentially as a diagnostic tool in most *Anaplasma*-related epidemiological studies in Nigeria. This molecular based study reveals the first 16S ribosomal RNA based evidence of the Anaplasmosis in Bauchi state, Nigeria. Thisstudy employed the use of *Anaplasma*/*Ehrlichia*universal primers to determine the molecular profiles of *Anaplasmaspp.* infecting cattle in the study area and analyzed the phylogenetic relationship and genetic diversity among the identified isolates and those from

different countries. To the best of our knowledge, this is the first phylogenetic analysis of *Anaplasma spp.*circulating among cattle population in thearea.

2. MATERIALS AND METHODS

2.1 Ethical Approval

Ethical approval was obtained from the Animal use and care committee (AUCC) of the National veterinary Research institute Bauchi state. An introductory letter was collected from the Biological Sciences department of the ATBU and taken to the Abattoir before sample collection.

2.2 Study area

The study was conducted between the months of May 2021 to January 2022 at the Bauchi central abattoir. The Bauchi Central abattoir is the largest abattoir in the state. It is about 6 kilometres away from the state capital and situated along Gombe Adamawa road in North Eastern Nigeria. The animals to beslaughtered are bought by butchers from nearby villages and towns markets. The abattoir has an average daily sacrifice of 50 cattle. The state lies between Latitude 9.3° and 12.3° North of the Equator and Longitude 8.5° and 11° East of the Greenwich meridian. The state covers a total land area of 49,259.01sqkm which is about 5.3% of Nigeria's total land mass. It has a population of 4, 676,465 people based on the 2006 population census. Bauchi state is one of the states in the Northern part of Nigeria that spans two distinctive vegetation zones namely the Sudan savanna and the Sahel savanna.

2.3. Sample collection

Samples were collected at the point of slaughter from the severed jugular vein into the Ethylene Diamine Tetra Acetate (EDTA) bottles which were immediately labelled and placed in a box containing ice packs. Each sample was assigned a Laboratory Identification Number. The blood samples were collected between the months of May 2021 to January 2022 at the Bauchi central abattoir. Both sexes of cattle were considered for the purpose of this research. The blood samples were transported to the National Veterinary Research Institute Vom for analysis. Samples were subjected to routine microscopy and positive samples obtained were stored at -20℃ pending further analysis. The labelled blood samples were stored at -20℃ pending DNA extraction.

2.4 DNA Extraction

DNA extraction was carried out using the Quick -DNA™ Miniprep kit catalogue Nos. D3024 & D3025 using the Quick protocol. All procedures were performed at room temperature (15-30℃) and all centrifugation steps were performed at 10,000xg-16,000xg using Eppendorf centrifuge 5417R. For optimal performance, beta-mercapto-ethanol was added to the Genomic lysis buffer to from a final dilution of 0.5% (V/v) i.e. 250µl per 50ml.To lyse the sample, 4 volumes of Genomic Lysing buffer (800µl) was added to 200µl of the sample (whole blood) in a volume of 4:1. Samples were vortexed for 4-6 seconds (to break down the cells) and then allowed to stand at room temperature for 5-10 minutes.The mixture was then transferred to a zymo-spin™ column in a collection tube and centrifuged at 12,000xg for 1 minute and then the collection tube with the flow through was discarded. Zymo-spin™ column was then transferred to a new collection tube and 200µl of DNA Pre-wash buffer was added to the spin column and was centrifuged at 12,000xg for 1 minute. A 500µl of g-DNA Wash buffer was added to the spin column and it was centrifuged at

12,000xg for 1 minute.The spin column was then transferred to a clean microcentrifuge tube. 50µl DNA Elution buffer was added to the spin column and was added incubated for 5 minutes at room temperature and then centrifuged at top speed of 16,0000xg for 30 seconds to elute the DNA. The eluted DNA was stored at -20℃ pending to next analysis.

2.5 PCR Amplification of 16S rRNA

Extracted DNA from the blood sample was amplified with specific primers targeting forward and reverse primers targeting the 16S rRNA (≈430 bp) genes in the *Anaplasma*parasite as described by [12]. PCR was performed using BioinGentech Veterinary PCR kits (Concepcion, Chile) according to the instructions of the manufacturer. The primary PCR was performed with *Anaplasma/Ehrlichia* universal primers kit.

Table 1: Oligonucleotide sequence of the primers used for Amplification of 16sRNA gene

Genes	Primers	Oligonucleotide sequence	Amplicon size	Reference
16S rRNA	F R	GGTTTAATTCGATGCAACGCGA CGTATTACACCGTGGCATG	**430**	**Simuunza, 2009**

The PCR amplification followed the cycling conditions by initial denaturation at 94℃ for 2 minutes, 30 cycles (94℃ for 30seconds, 57℃ for 30 seconds, 72℃ for 60 seconds) and final extension at 72℃ for 5 minutes.

2.6 Quantification of PCR products by Agarose Gel Electrophoresis

The buffer used for elution in the subsequent steps were equilibrated at room temperature (15-25℃) and the water bath was heated to 56℃, 2% agarose gels was used for electrophoresis [13].

2.7 Genes Sequencing Analysis

Amplified DNA fragments of the 16sRNA gene from the *Anaplasma* spp. isolates were directly sequenced using the Sanger method, and the analyzed sequences were compared by BLASTn with those present in the GenBank database.

2 RESULTS

3.1 Molecular characteristics of the *Anaplasma species*detected from cattle in the study area

Polymerase Chain Reaction (PCR) was used to characterize the pathogens observed in the microscopy. DNA extracted from the blood sampleswere analysed and 52% of the samples were found to be positive for 16SrRNA in the *Anaplasma species*with band corresponding to 430 bp, as shown in the reference primers(Figure 1).

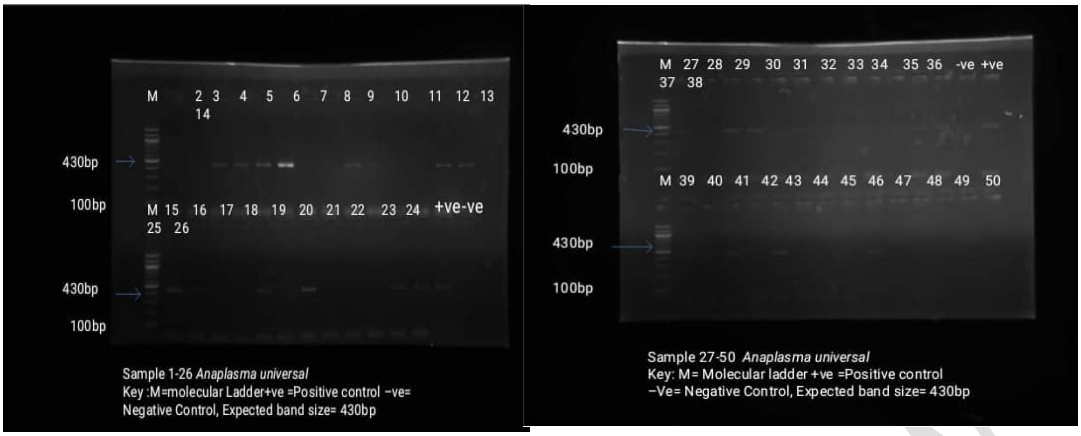

Figure 1:Agarose gel image of PCR products(16SrRNA) of *Anaplasma species* with amplicon sizeof 430bp

3.2 Nucleotide and Phylogenetic analysis

Amplified DNA fragments of the 16sRNA gene from the *Anaplasmaspp.* isolates were directly sequenced using the Sanger method.The 16S rRNA nucleotide sequences obtained in this study were edited manually using the Bioedit version 7.0.5.3 [14] and were compared to reference sequences available in the GenBank using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Ten reference 16S rRNA sequences of different *Anaplasmaspp*. were retrieved from GenBank and added to alignments of the sequences of the study. Phylogenetic analysis was inferred using the Neighbour-Joining method [15]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed [16]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [16]. Evolutionary analyses were conducted in MEGA11 [17].

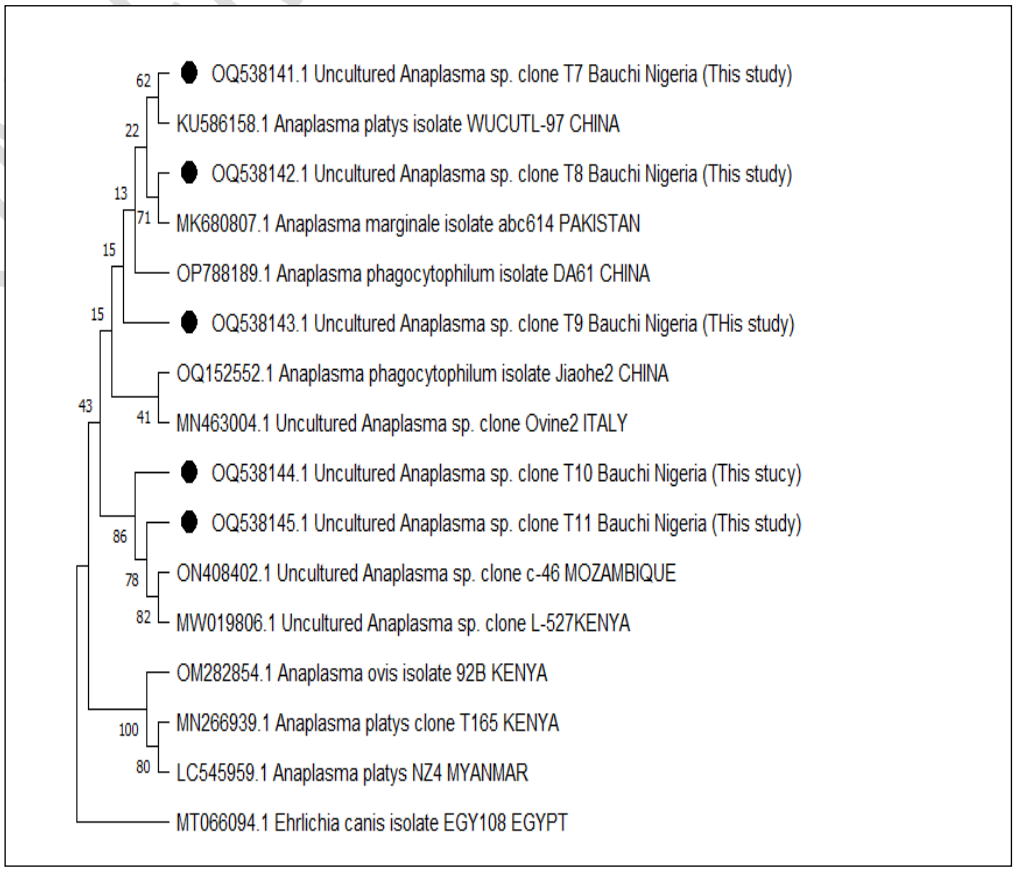

Fig 2. Phylogenetic tree based on the 16S rRNA nucleotide sequence of *Anaplasmaspp.* of cattle from Bauchi state, Nigeria. The tree was inferred using the Neighbour-Joining method [15]. The evolutionary distances were computed using the Kimura 2-parameter method [18] and are in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 64 positions in the final dataset. Sequenced obtained in this study were identified by a black circle (●). Nucleotide sequence of *Ehrlichiacanis* (MT066094.1) from Egypt was used as an outgroup.

3.3 Nucleotide sequence accession numbers for Anaplasma detected in cattle in Bauchi state

The partial 16SrRNA gene sequences obtained from this study were deposited in the Genbank. The gene sequences were deposited under accession numbers OQ538141 for *Anaplasma platys*, OQ538142 for *Anaplasmamarginale*, OQ538143 for *Anaplasmaphagocytophilum* and OQ538144 to OQ538145 for uncultured Anaplasma*species*described as isolates T7, T8, T9, T10 and T10 respectively (Table 2).

Table 2: *Anaplasma spp.* detected by BLASTn analysis of 16SrRNA gene sequences of the isolates from Bauchi state

Isolate	Accession no. (this study)	Matching sequence	Accession no. highest match	E-value	% Identity
T7	OQ538141.1	*Anaplasma platys*	KU586158.1	0.0	>60%
T8	OQ538142.1	*Anaplasmamarginale*	MK680807.1	0.0	
T9A	OQ538143.1	*Anaplasmaphagocytophilum*	OP788189.1, OQ152552.1	0.0	
T10, T11	OQ538144.1, OQ538145.1	*Uncultured Anaplasma species*	ON408402.1, MW019806.1	0.0	

3 DISCUSSION

A total of 50 blood samples from the cattle positive for *Anaplasma species* through microscopy were screened by PCR for the presence of *Anaplasma* parasite using primers targeting the 16SrRNA gene. *Anaplasma* organism was detected in 26 blood samples from the cattle when primers targeting the 16SrRNA was used.The overall positive rate was 52%.

The 16S rRNA nucleotide sequences of *Anaplasmaspp.* obtained in this study were clustered into four groups following phylogenetic analysis. The sequence OQ538141.1 clustered with the sequence of *Anaplasma platys* from China in the GenBank with a high bootstrap value (>60%). Similarly, another sample, OQ538142.1 clustered with the sequence of *Anaplasmamarginale* (MK680807.1) from Pakistan. Two sequences from this study, OQ538144.1 and OQ538145.1 were placed in the same clade with Uncultured *Anaplasmaspp.* from African countries, Mozambique (ON408402.1) and Kenya (MW019806.1). One sequence OQ538143.1, from this study formed a distinct clade and was placed between two *Anaplasmaphagocytophilum* sequences (OP788189.1, OQ152552.1) from China (Fig. 2).

Previous studies conducted using 16SrRNA gene detected *Anaplasma* organisms in ruminants from China by PCR [19][20] is ~~in~~consistent with the findings of this study. However, most of the positive samples were detected by PCR for 16srRNA gene. This detection of DNA is an indication that *Anaplasma* parasite is present in cattle in Bauchi state. The result shows that cattle are exposed to ticks infected by the various *Anaplasma species*. This suggests that 16SrRNA can be said to be sensitive for the detection of *Anaplasma species*.[21] confirmed the presence of *Anaplasma species* in cattle by BLASTn analysis after the sequencing of the 16SrRNA gene. In this study, the BLASTn analysis of the 16SrRNA gene fragments revealed nucleotides homologous to those of *A. phagocytophilum* and *A.platys* from cattle samples. This finding indicates infection of cattle with *A. phagocytophilum* and *A.platys*in the study area. It is well known that *A. centrale and A. marginale* are the main *Anaplasma species* that infect cattle in Nigeria. However, the findings from this study have shown that cattle in Bauchi state can also contain various *Anaplasma species* including the zoonotic ones like*A. phagocytophilum*. This result is ~~in~~ consistent with the studies of [21] and [22] in other countries who found *A. phagocytophilum* to infect cattle.

*A.platys*was also detected in the cattle from this study and this implies that cattle could be potential hosts for various *Anaplasma*isolates different from the previous studies that cattle in the state are only infected with *A. marginale* and *A.centrale*and this is the first report of *A. phagocytophilum* and *A.platys*infecting cattle in Bauchi state even though there are other studies from Nigeria where they were also found.The discovery of *A. phagocytophilum* in cattle calls for further studies to determine its public health implications because it is a zoonotic pathogen and ~~so it~~ haspublic health implications.

4 CONCLUSION

The result from this study indicates that*Anaplasma species* from Bauchi state clustered with other*Anaplasma* sequences and based on multiple alignment, the sequence presented nucleotide differences between them suggesting that different strains are circulating in the state based on the analyzed data.DNA sequencing results of the 16SrRNA also revealed that *Anaplasma platys* and

*Anaplasmaphagocytophylum*are circulating among cattle herds in the state and this is of public health implication because *A. phagocytophilum* is zoonotic. Five nucleotide sequences were obtained from this study and deposited in Genbank under the following accession numbers: OQ538141 for *Anaplasma platys*, OQ538142 for *Anaplamamarginale*, OQ538143 for *Anaplasmaphagocytophilum* and OQ538144 to OQ538145 for uncultured *Anaplasma species.*

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