Comparism of Microscopy and molecular diagnosis of *Ehrlichiaruminantium* in cattles in Makurdi Benue Nigeria.

Abstract

Introduction:Makurdi, is the capital city of Benue State, the food basket of the Nigeria, the city challenged with tick-borne haemoparasite of cattle that are responsible for severe losses caused either by negative impact of ticks blood loss or blood related infections, damage to hides and others.

Aim: This study was designed to identify and Characterize of Tick-borne Haemoparasite of Cattle within Makurdi Nigeria using microscopy and molecular techniques (PCR).

Methodology: Blood sample were collected from a total of 432 cattle of both sexes and analyzed microscopically using thin blood film and DNA examination was done using polymerase chain reaction (PCR). Tick-borne pathogens were identified and characterized by PCR amplification using species specific primer of 16s rRNA for *Ehrlichia*. Data obtained were analyzed using chi square, t-test and P values at less than 0.05 were considered significantly different.

Results: The result of comparison of prevalence of infection of haeomoparasite using microscopy and PCR revealed that, microscopy was not able to detect *Ehrlichia* while PCR yielded at percentage of 15%, *Ehrlichia*prevalence. The percentage of prevalence of haemoparasite was highly detected by PCR than microscopy and this was statistically significant ($P < 0.05$).

Conclusion: Tick – borne haemoparasite in cattle have been seen to be prevalent in Makurdi metropolis and molecular method such as PCR can effectively diagnose the infection

Key words: Diagnostic methods Molecular, Microscopy, *Ehrlichia*, Cattle.

Background

Ticks, being haematophagous are capable of transmitting disease agents such as viruses, bacteria and protozoa. Historically, they are considered second, only to mosquitoes, in their ability to transmit disease agents (Salih, 2015). Ticks attach to their hosts and facilitate transmission of infectious agents to different geographical regions via traveling pets, migration of animals or other means of transportation (Stephanie, 2017). One of the major problems of the livestock industries have been tick borne diseases. Often time some

of the diseases appear very difficult to diagnose, mainly by microscopy. Makurdi Benue Nigeria has many livestock farmers who are faced with the challenge of one or more livestock disease. Most of these diseasesare difficult to diagnoseusing microscope, hence the need to use PCR diagnostic method.

Sample analytical procedure

The blood samples were collected using standard procedure and wereanalyzed using microscopic method (Thin blood film method) and polymerase chain reaction (PCR)

Sample preparation for PCR

Samples were treated with lysis buffer and Proteinase K to remove potential inhibitors of PCR present in the blood (Zerihun *et al.,* 2017).

Sample analytical procedure for PCR

The tick-borne pathogens were identied and characterized by PCR amplification using species specific primers for different pathogens. The forward and reverse primers which are species specific primers for PCR amplification of 16s rRNA (*Ehrlichia*) were used (Table 1). All PCR reactions were conducted using New England Biolab PCR reagentAmplitaq Gold® 360 reagent. The amplification was performed in an automated thermo-cycler with an initial denaturation step at 95 ^{o}C for 3 min. followed by 30 cycles at 94 ^{o}C for 30 s, 59 ^{o}C for 1 min and 72 ^{o}C for 1 min, annealing s at 55 ^{o}Cfor45 minutes with a final extension step of 72 ^{o}C for 5 min. The resulting amplified products was electrophoresed on a 2 % agarose gel at 95 V for 45 minutes, stained with ethidium bromide molecular weight marker was included to identify the size. The amplified product was visualized under a UV transilluminator and photographed with a figured camera. (Zerihun *et al.,* 2017).

Table 1: List of universal and species specific primers used for PCR amplification of

16s rRNA (Ehrlichia)

Parasite Speciese Primer	Primer Sequence	Amplian size (bp)
Ehrlicha, Universal AnE – F AnE – R	*F:5^1_ GGTTTAATTCGATGCA ACGCGA – 3'R;5 – CGTAT TCACCGTGGC ATG – 3^1*	*430*
Species specific		
E. ruminantium ER – R	*R:5^1_ GAGTGCCCAGCATTA CCTGT – 3*	*201*

Statistics

Data obtained was analyzed using Chi-square test, to determine whether there is a significant different between the expected frequencies and the observed frequencies in one or more categories and to examine differences within categorical variables. PCR was compared with the diagnostic performance of microscopy by calculating sensitivity, specificity, positive predictive value and negative predictive value.

Sensitivity is calculated as

$$\frac{\text{Number of true positive}}{\text{Number of true positives + Number of false negatives}}$$

Specificity is calculated as

$$\frac{\text{Number of true negative}}{\text{Number of true negative + Number of false positive}}$$

Positive predictive value =

$$\text{Positive predictive value} = \frac{\text{Number of true positive}}{\text{Number of true positive} + \text{Number of false positive}}$$

Negative predictive value =

$$\text{Negative predictive value} = \frac{\text{Number of true negativ}}{\text{Number of false negative} + \text{Number of true nagetive}}$$

Results

The result of diagnosis of *Ehrlichia ruminatium* with microscopy using PCR is presented in Table 2. The sensitivity of PCR to *Ehrlichia ruminatium* yielded 0.0 % estimated value and 0 % lower limit on 95 % confidence interval but 37.1 % upper limit.

The specificity produced 1 % estimated and at lower limit PCR yielded 1 % specificity and 91.3 % at upper limit. However, there was no case of PPV at estimated, lower and upper limit on 95 % confidence interval. There was high record of NPV of 85 % estimated and 72.9 % lower limit and 92.5 % upper limit.

Table 2: Diagnostic parameters of Ehrlichia ruminantium with microscopy using PCR as reference test

Ehrlichia ruminantium	Estimated Value %	95% confidence Interval	
		Lower limit %	Upper limit %
Sensitivity	*0*	0	37.1
Specificity	*1*	1	91.3
PPV	*Nil*	Nil	Nil
NPV	*85*	72.9	92.5

Plate 1 shows the representation of agarose-gel electrophoresis result showing primary reaction of *Ehrlichia ruminantium* positive samples amplified at 430 base pairs as shown by the white thick band between 400 base pair and 500 bed pairs. The DNA ladder reads at lane 1, 2, indicate Negative sample of *Ehrlichia ruminantium* and lane 3, 4, 5, 6, 7, 8, 9, 10 11 indicate positive cattle sample. Lane 12 indicates control.

Plate 2 is representation of agarose-gel electrophoresis result showing secondary reaction of *Ehrlichia ruminatium* positive sample amplified at 201 bed pairs as shown by the thick white band between 200 bed pair and 300 bed pair. The DNA ladder reads at lane 1, 2, 3, 4, 5, 6 which indicate positive samples of *Ehrlichia ruminatium* in cattle and lane 8 indicate negative sample. Lane 9 indicates control.
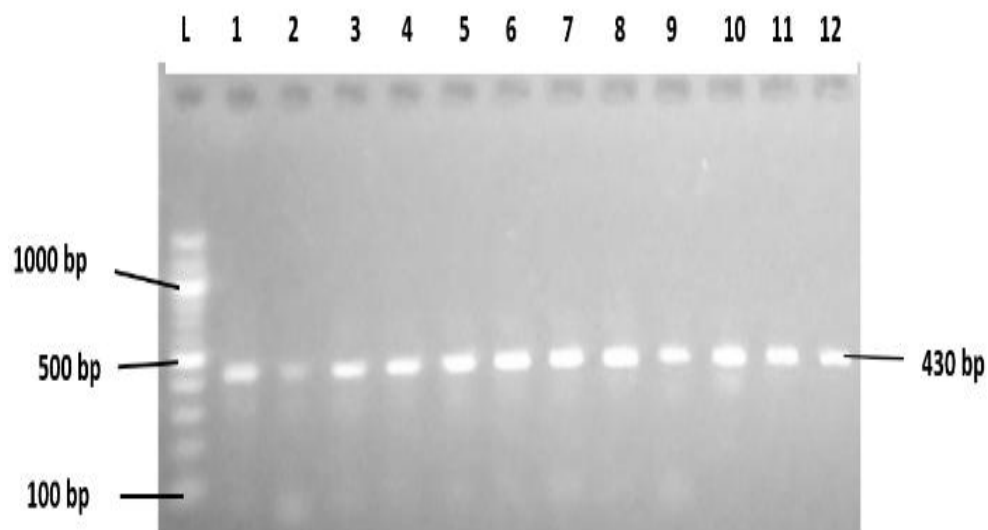

Plate 1: Representation of agarose-gel electrophoresis result showing Primary reaction of
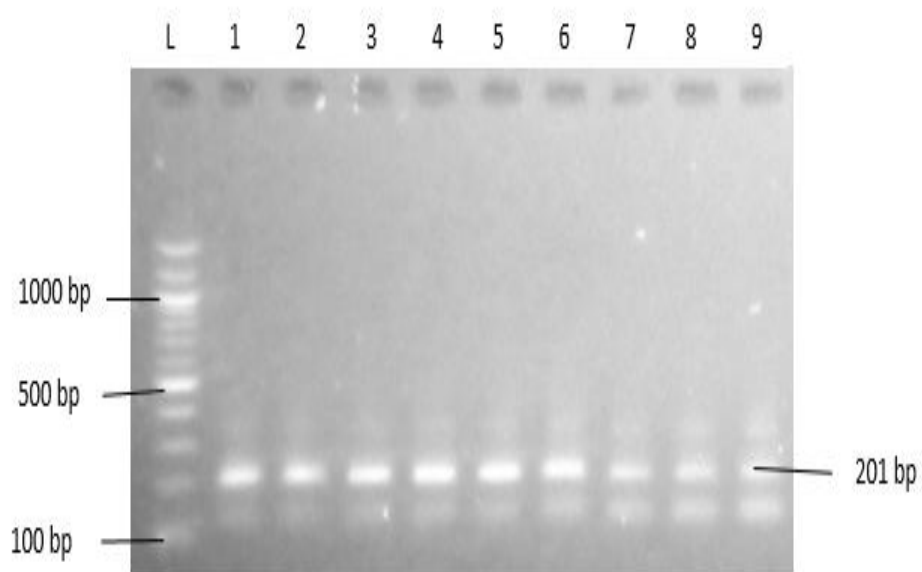
Ehlrichia species 430 bp fragment

Plate 2: Representation of agarose-gel electrophoresis result showing Secondary reaction of Ehrlichia ruminatium lower panel = 201 bp fragment.

Discussion

PCR method was able to detected *Ehrlichia ruminantum* which was not detected microscopically. This is an indication that false positive sample and false negative sample have been recorded using microscopy as Vahid (2014) and Abanda *et al.,* (2019) stated that microscopy is most used for the identification of piroplasma and it accompanied with some technical problems which leads to false morphological diagnosis. Thus, this method is not sensitive enough or sufficiently specific to detect chronic infection of cattle with heart water diseases.

The study revealed the presence of *E. ruminantium*. However, in Nigeria particularly Makurdi, identification and characterizationof tick-borne haemoparasitic diseasesusing molecular method (PCR)are scarce however, using microscopy, many researchers have reported the occurrence of *E. ruminantium* in cattle and other ruminant. Stuen *et al*., 2013, Zhou *et al.,* 2016 reported that heartwater disease can result in public health and economic consequences to cattle rearers.

This study used the same species specific primers used for PCR amplificatuion of 16s rRNA genes for *Ehrlichia ruminantium* as used by Jalali*et al.*(2013) for detecting pathogens by PCR.

Thus, the species specific primers have been used for detection and identification of both plant and animals pathogens by PCR hence this indicate it could be widely used to develop molecular techniques for defection of other pathogens of veterinary and medical importance (Happi *et al.,* 2020). Undoubtedly from the literature, these appear to add to the baseline data in which primers are designed using species specific gene for the detection of tick-borne haemoparasites.

The 16s rRNA gene sequence primer for Ehrlichia from this study shared high identity (100%) with samples collected from cattle. 16s rRNA gene fragment for Ehrlichia was bonds at approximately 201 bp for Ehrlichia. This result is in line with the study conducted by Aaron *et al*. (2019) who also reported detectionof pathogenic*Theileria, Anaplasma* and*Ehrlichia*species on their study on Molecular detection and genetic characterization of pathogenic *Theileria, Anaplasma* and *Ehrlichia* species among apparently healthy sheep in central and Western Kenya. Many reports have documented these gene markers to be used for understanding and of the molecular epidemiology of bovine *Ehrlichia* species and other diseases associated with tick-borne haemoparasite (Ybanez *et al*., 2016; Wei *et al*., 2016). The result reveals the high sensitivity and specificity of PCR in identification and characterization of tick-borne haemoparasite. Therefore, PCR is reported to be characterized by high specificity. Specificity and sensitivity thus being able to identify *E. ruminantium* from other species of tick-borne haemoparasites which was not detected by microscopy.

In comparison of molecular tools to microscopic analyses of blood smears, blood microscopy is used for rapid diagnosis and informative purposes on animals health statutes, and again, identification by microscopy is prone to errors in species identification, as pathogen may look very similar among and between genera leading to misidentification, or may be missed depending on the animals patency or developmental status.

In Comparison between Polymerase chain reaction (PCR) and Microscopy, PCR yielded higher sensitivity and specificity in identification of tick-borne haemoparasite than microscopic diagnostic methods. This could be because PCR methods are gene specific and the primers are developed for particular species. It is an indication that PCR probability has a higher ability in detecting true negative samples.

The relatively higher detection rate with 16S rRNA gene as compared to microscopy suggest that the marker is highly sensitive and specific for *Ehrlichia* detection. PCR diagnostic method was able to detect *E. ruminantium*while microscopy was not able to identifythe organism due to low sensitivity. Sex, age, breed and location may affect the infection of bovine*Ehrlichia.*

Conclusion

Conclusively, PCR diagnostic method is more sensitive than microscopy in the detection of *E. ruminantium.* Therefore, microscopy alone is not okay to be used in diagnosis of *E. ruminatium*as it can give a false negative result.

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