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

Abstract

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. This study was designed to identify and Characterize of Tick-borne Haemoparasite of Cattle within Makurdi Nigeria using microscopy and molecular techniques (PCR). 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 Erilcdia. Data obtained were analyzed using chi square, t-test and P values at less than 0.05 were considered significant different. 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 ($P < 0.05$). Tick – borne haemoparasite in cattle have been seen to be prevalence in Makurdi metropolis and molecula method such as PCR can effectively.

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 diagnosed, 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 disease appears negative when diagnosed using microscope, hence the need to use PCR diagnostic method.

Sample analytical procedure

The blood sample were collected using standard procedure and was analysis 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 (Ehrillchia) was use (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 45 s at 55 ^{o}C 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 at 100 bp molecular weight masses was included to identify the bed weight. 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 Sequence	Amplian size (bp)
Primer		
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 = $\frac{\text{Number of true positive}}{\text{Nunmber of true positive + Number of false positive}}$

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

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 bed pairs as shown

by the white thick band between 400 bed 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 positive and negative 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 positive and negative control.

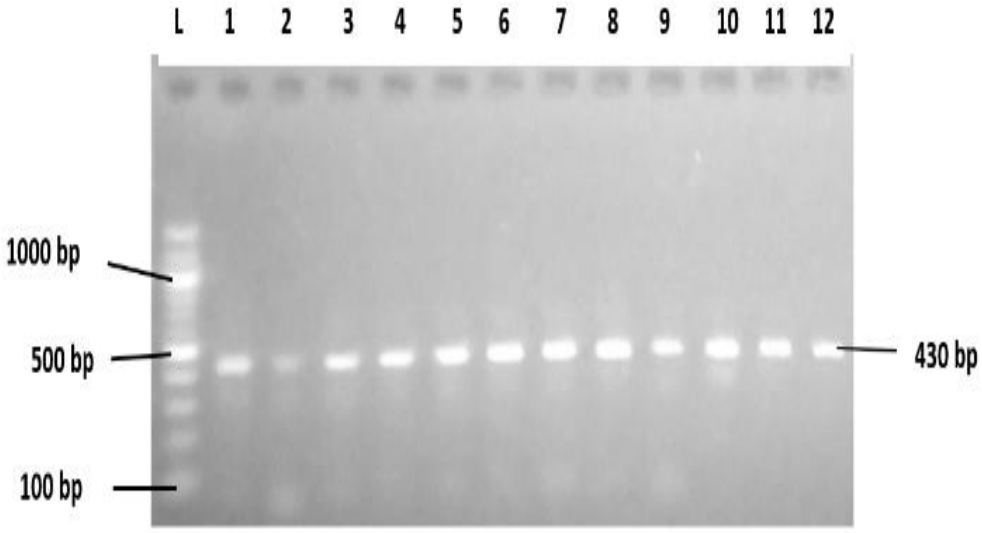

Plate 1: Representation of agarose-gel electrophoresis result showing Primary reaction of Ehlrichia species 430 bp fragment

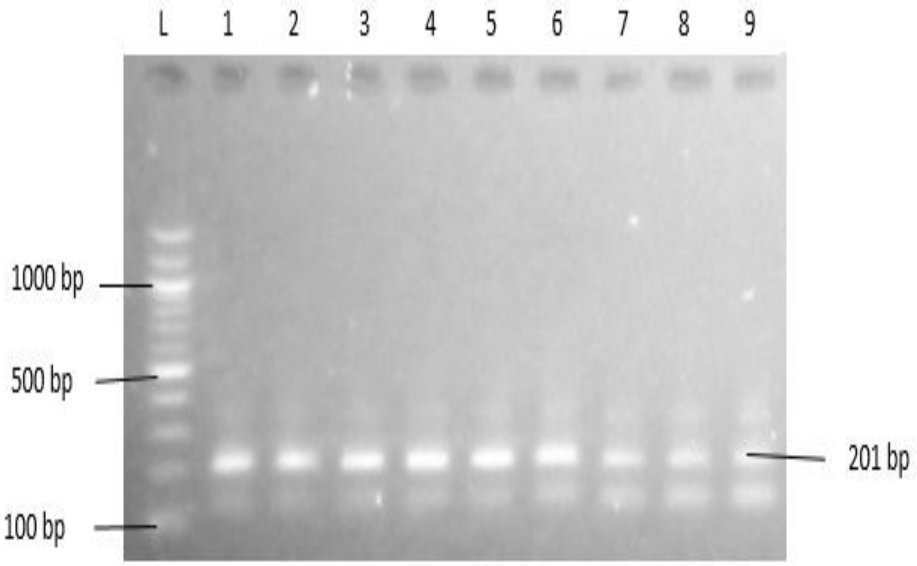

Plate 2: Representation of agarose-gel electrophoresis result showing Secondary reaction of Ehrlichia ruminatium lower panel = 201 bp fragment

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 cattle with infected with heart water diseases.

The study revealed the presence of *E. ruminantium*. However, in Nigeria particularly Makurdi, using molecular method (PCR) to identification and characterization tick-borne haemoparasitic diseases are scarce, on microscopy, many literatures have reported the occurrence of these 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 has generated species specific primers used for PCR amplificatuion of 16s rRNA genes for *Ehrlichia ruminantium*. This however corroborated with the approach of Jalali *et al.,* 2013 who used same synthesized primers 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 detection of pathogenic Theileria, Anamplasa and Ehrlicha species on their study on Molecular detection and genetic characterization of pathogenic Theileria, Anamplasa and Ehrlicha species among apparently healthy sheep in central and Western Kenya (Aaron *et al*., 2019). Many reports have documented these gene markers to be used for understanding and of the molecular epidemiology of bovine Ehrlicha species and other diseases associated with tick-borne haemoparasite (Ybanez *et al*., 2016; Wei *et al*., 2016). This 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 identified *E. ruminantum* from other species of tick-borne haemoparasites which was unable to be detected by microscopy.

In comparison of molecular tools to microscopic analyses of blood smears, blood microscopy is used for rapid diagnostic 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 Microscopic, 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 of 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. ruminatium* which microscopic was not able to identify due to low sensitivity. Sex, age, breed and location may affect the infection of bovis Ehrlichia.

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