

Comparism of Microscopy and molecular diagnosis of *Ehrlichia ruminantium* 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 *Ehrlichia*. Data obtained were analyzed using chi square, t-test and P values at less than 0.05 were considered significantly different. The result of comparison of prevalence of infection of haemoparasite 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 prevalent in Makurdi metropolis and molecular method such as PCR can effectively diagnose the infection.

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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 are difficult to diagnose ~~appears negative when~~
~~diagnosed~~ using microscope, hence the need to use PCR diagnostic method.

Sample analytical procedure

The blood samples s were collected using standard procedure and ~~was~~ were analysed ~~is~~
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 identified 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 reagent
Amplitaq Gold® 360 reagent. The amplification was performed in an automated thermo-
cycler with an initial denaturation step at 95 °C for 3 min. followed by 30 cycles at 94 °C
for 30 s, 59 °C for 1 min and 72 °C for 1 min, annealing ~~45 s~~ at 55 °C for 45 s with a final
extension step of 72 °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 ~~and at 100 bp~~
molecular weight marker masses was included to identify the ~~band weight~~ band 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 Species	Primer Sequence	Amplian size (bp)
Primer		
Ehrlichia, Universal AnE – F AnE – R	<i>F:5'</i> _ <i>GGTTTAATTCGATGCA</i> <i>ACGCCA – 3'R;5 –</i> <i>CGTAT TCACCGTGGC</i> <i>ATG – 3'</i>	430
Species specific		
E. ruminantium ER – R	<i>R:5'</i> _ <i>GAGTGCCCAGCATTA</i> <i>CCTGT – 3</i>	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
$$\frac{\text{Number of true positive}}{\text{Number of true positives} + \text{Number of false negatives}}$$

Specificity is calculated as
$$\frac{\text{Number of true negative}}{\text{Number of true negative} + \text{Number of false positive}}$$

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

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

The result of diagnosis of *Ehrlichia ruminantium* with microscopy using PCR is presented in Table 2. The sensitivity of PCR to *Ehrlichia ruminantium* 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

<i>Ehrlichia ruminantium</i>	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~~-base pairs as shown by the white thick band between 400 ~~base~~~~bed~~ pair and 500 ~~base~~~~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.

Comment [R1]: How a lane contain both negative and positive control. Please correct.

Plate 2 is representation of agarose-gel electrophoresis result showing secondary reaction of *Ehrlichia ruminantium* 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 ruminantium* in cattle and lane 8 indicate

negative sample. Lane 9 indicates positive and negative control.

Comment [R2]: How a lane contain both negative and positive control. Please correct.

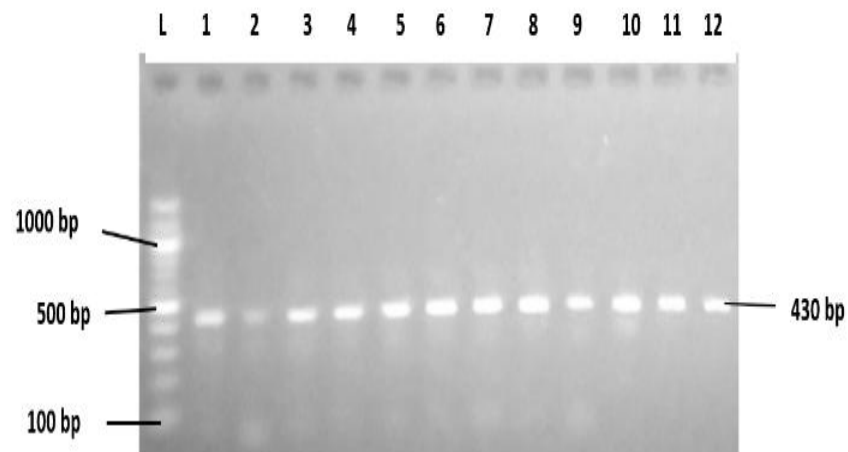


Plate 1: Representation of agarose-gel electrophoresis result showing Primary reaction of *Ehlichia* species 430 bp fragment

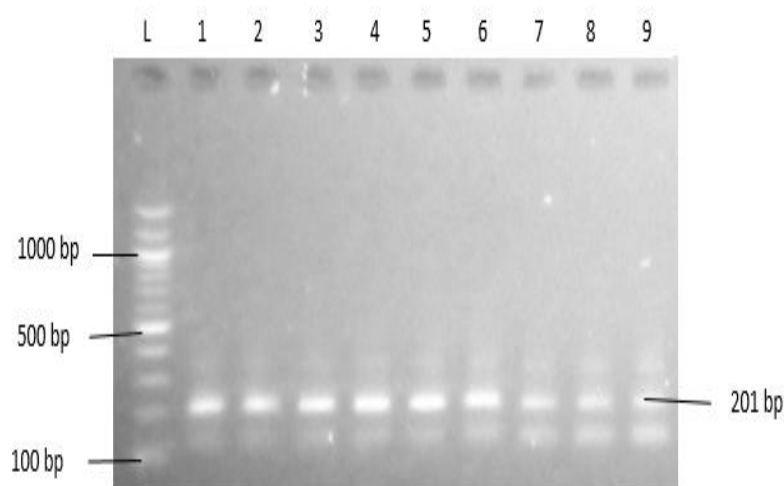


Plate 2: Representation of agarose-gel electrophoresis result showing Secondary reaction of *Ehrlichia ruminantium* lower panel = 201 bp fragment

PCR method was able to detected *Ehrlichia ruminantium* 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 infected~~ with heart water diseases.

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The study revealed the presence of *E. ruminantium*. However, in Nigeria particularly Makurdi, ~~using molecular method (PCR) to~~ identification and characterization of tick-borne haemoparasitic diseases using molecular method (PCR) are scarce ~~however, on~~ using microscopy, many ~~literatures-researchers~~ 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.

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This study has generated species specific primers used for PCR amplification 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.

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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 detection 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 ~~bands~~ 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*, *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 (~~Aaron et al., 2019~~). 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~~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 identify ~~ied~~ *E. ruminant*ium from other species of tick-borne haemoparasites which was ~~unable to be~~not detected by microscopy. In comparison of molecular tools to microscopic analyses of blood smears, blood microscopy is used for rapid diagnosis~~istie~~ 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~~ie~~, 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~~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. ruminant*ium wh~~ile~~ich microscopy~~ie~~ was not able to identify the organism due to low sensitivity. Sex, age, breed and location may affect the infection of bovi~~nes~~ *Ehrlichia*.

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References

- Aaron, E. R., Gabriel, O. A., Paul, F., Adjou, M., Seung, H. L., Charoonluk, J., Mingming, L., Yang, G., Huanping, G., Weiqing, Z., Arternis, E., Eloiza, M., Jixu, L., Oriel, T., Noboru, I., Hiroshi, S. and Xuenan, X. (2019). Molecular detection and genetic characterization of pathogenic *Theileria*, *Anaplasma* and *Ehrlichia* species among apparently healthy sheep in central and Western Kenya. *Onderstepoort Journal of veterinary research*. 86 (1): 1839 – 1847
- Happi, A. N., [Osifade](#), O., [Oluniyi](#), P. E. and [Ogunro](#), B. N. (2020). Comparison of Light Microscopy and Polymerase Chain Reaction for the Detection of Haemoparasites in Cattle in Nigeria. *Acta Parasitologica*. 65: 44–56.
- Salih, D. A., El Hussein, A. M. and Singla, L. D. (2015). Diagnostic approaches for tick-borne haemoparasitic diseases in livestock. *Journal of Veterinary medicine and Animal Health*. 7 (2): 45-56.
- Stephanie, L. R., Ricky, L., Charles, S. A. and Elizabeth, W. (2017). Do Tick Attachment Times Vary between Different Tick-Pathogen Systems. *Journal Environments*. 4, (3): 7-13.
- Stuen, S. (2013). Tick-borne infections in small ruminants in northern Europe. Ticks and Tick-borne Diseases. 110, (3); 142-144.
- Wei, F., Song, M., Liu, H., Wang, B., Wang, S., Wang, Z., Ma, H., Li, Z., Zeng, Z., Qian, J., Liu, Q. (2016). Molecular Detection and Characterization of Zoonotic and Veterinary Pathogens in Ticks from Northeastern China. *Frontiers in Microbiology*. 7:1913-1919.
- Ybañez, A. P., Ybañez, R. H. D., Yokoyama, N., and Inokuma, H. (2016). Multiple infections of *Anaplasma platys* variants in Philippine dogs. *Vet World*. 9 (12): 1456–1460.
- Zerihun, H., Krücken, J., Baumann, M., Ahmed, J. S., Clausen, P. H. and Nijhof, A. M. (2017). Molecular detection of tick-borne pathogens in cattle from Southwestern Ethiopia. *PLoS ONE* 12(11): 43-49.
- Zhou, M., Cao, S., Sevinc, F., Ceylan, M. C., Franck, P., Moumouni, A., Jirapattharasate, C., Liu, M., Wang, G., Iguchi, A., Vudriko, P., Suzuki, H., Xuan, X. (2016). Molecular detection and genetic identification of *Babesia bigemina*, *Theileria annulata*, *Theileria orientalis* and *Anaplasma marginale* in Turkey. *Ticks and Tick-borne Diseases*. 7 (1): 126-134