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# Molecular diagnosis of the tick-borne pathogen *Anaplasma marginale* in cattle blood samples from Nigeria using qPCR.

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## *Abstract*

Tick-borne diseases (TBDs) are some of the most important animal health and management problems in Africa, including Nigeria. This study aims to determine the prevalence of an important tick borne disease, anaplasmosis, in a North-central region of Nigeria. Blood samples were collected from cattle and stored on Whatman FTA® cards. Information on village, age and sex associated with each cattle was also recorded. The packed red blood cell volume (PCV) for each blood sample was determined. After DNA extraction, pathogen presence was evaluated by TaqMan® based qPCR of which 75.9% of the cattle tested positive for *Anaplasma marginale*. Statistical analysis revealed that the presence of *A. marginale* infection differed significantly between cattle age groups. However, there was no significant difference in the prevalence of this pathogen between the sexes or among cattle grouped by PCV level. Finally, using a highly sensitive molecular method our pioneer study contributes to the improvement of the current knowledge regarding tick-borne pathogens that seriously affect animal health in specific areas of Nigeria.

## *Keywords*

*Anaplasma marginale;* tick-borne diseases; tick-borne pathogens; qPCR; Kwara; Nigeria.

## *Abbreviations*

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

LGA: Local Government Area

PCR: Polymerase chain reaction

PCV: Packed cell volume

qPCR: Real time PCR

rRNA: Ribosomal ribonucleic acid

TBDs: Tick-borne diseases

## *Introduction*

The interest in tick-borne diseases (TBDs) has emerged during the past decades, especially from the veterinary point of view (Jongejan and Uilenberg, 2004). It is well known that the prevalence of these diseases around the world is gradually increasing, demanding new treatment and preventive control measures (Dantas-Torres et al., 2012). In Africa, TBDs are one of the most important animal health and management problems, seriously affecting the development and productivity of the livestock industry (Young et al., 1988). It has been estimated that the annual cost of TBDs to the development and productivity of this industry is between US$ 13.9-18.7 billion based on a worldwide cattle population of 1288 million (de Castro, 1997). Other sources of financial losses that are more difficult to estimate also associated with TBDs include the downgrading of hides due to tick bites, secondary infections due to dermatophilosis and reduced fertility and abortion (Kivaria, 2006). In addition, some TBDs are also zoonotic and fatal to man (Mehlhorn and Schein, 1993). An important TBDs that pose a significant threat to cattle is bovine anaplasmosis whose transmission has been associated with ticks of the genus *Rhipicephalus* subgenus *Boophilus* (Aiello, 1998; Bock et al., 2004). This disease represent a major constraint to livestock productivity in the tropical and sub-tropical regions of the world (Jongejan and Uilenberg, 2004) and have been widely reported across Africa (Bell-Sakyi et al., 2004; Okuthe and Buyu, 2006; Gachohi et al., 2010; Simuunza et al., 2011), including in Nigeria (Kamani. et al., 2010; Reye et al., 2012). Bovine anaplasmosis is a tick-transmitted intraerythrocytic, rickettsial disease caused by *A. marginale* or *A. centrale* (Young et al., 1988). The disease is characterised by progressive anaemia due to destruction of erythrocytes by phagocytosis, resulting in icterus without haemoglobinemia and haemoglobinuria (Kocan et al., 2000). To the best of our knowledge, in Nigeria only a very few studies for the detection of tick-borne pathogens in cattle has used molecular methods and even less applied qPCR. The main objective of this study was to provide up-dated information using qPCR regarding the presence of *A. marginale* pathogens in blood samples collected from cattle of 11 villages of the Edu Local Government Area (LGA), in Kwara State, North-central Nigeria. A statistical analysis was also conducted to determine the effect of infection on cattle age, sex, levels of anaemia using packed cell volume (PCV). Our study constitutes the first report of the detection by qPCR of *A. marginale*

in cattle blood samples in Edu LGA, Nigeria. Through a highly sensitive molecular method, such as the qPCR TaqMan® based assay we expect to contribute for current knowledge about the circulating tick-borne pathogens that seriously affect animal health in some areas of Nigeria and for a better planning of effective tick control measures.

## *Materials and methods*

### *Study population*

Edu LGA was selected as the study location because it has very large pastoralist settlements and is one of the largest cattle producing area in Kwara State, North-central Nigeria. A local informant identified 11 cattle producing villages (Bacita, Belle, Bokungi, Fanagun, Fedudangi, Gonandogo, Mokwagi, Ndabata, Ndachewoye, Tshonga and Yelwa) in Edu LGA (Figure 1) and these formed the study population. Sixty-five households were visited in the 11 villages and between 3-4 cattle were sampled in each household. A total of 253 bovines were selected to collect individual blood samples between April and August 2013. During collection, the age and sex of each sampled animal was recorded.

### *Haematology*

Five millilitres (ml) of blood were collected from the jugular vein of each animal with a syringe, transferred into EDTA specimen container and kept on ice during transport to the laboratory facilities. From each sample, approximately 100 µl was applied into Whatman® FTA® cards (Whatman, UK). The cards were air dried and stored at room temperature, according to the manufacturer's instructions, for downstream applications. From the remainder of the collected blood, the packed cell volume (PCV) was also determined using the micro haematocrit method (McInroy, 1954).

### *DNA extraction*

Extraction of DNA from blood stored on Whatman® FTA® cards (Whatman, UK) was carried out following the method recommended for diagnosis of blood-borne infections (Ahmed et al., 2011), with some modifications. Briefly, a 3 mm diameter disc was cut from each blood sample stored on the Whatman® FTA® cards using a hand paper punch. To avoid cross contamination, five discs were punched from blank

filter paper between each sample and included as negative controls. The excised discs were first washed twice for 15 minutes with Whatman® FTA® Purification Reagent (Whatman, UK) in order to remove haemoglobin, and then washed twice for 15 minutes with 100 µl of Tris/EDTA buffer to remove the purification reagent. The discs were then dried for 30 minutes at 37°C. To elute the DNA, 60 µl of 5% aqueous suspension of Chelex Resin (Bio-Rad, UK) was added to each disc and incubated at 90°C for 30 minutes, as previously described (Becker et al., 2004). The eluted DNA was stored at -20 °C until used for the analysis.

### *Real time PCR*

Real time PCR was conducted to determine the presence of *A. marginale* using primers and TaqMan® fluorescence-labelled probes that specifically amplify a fragment of the *msp*1b gene of *A. marginale* (Carelli et al., 2007). PCR amplifications were carried out in a 7500 Fast Applied Biosystems thermocycler (Alfagene, Portugal) with the SensiFAST™ Probe Low-Rox kit (Bioline, Portugal). For the samples tested, a 20 µl reaction mix was prepared with 10 µl of SensiFAST™ Probe Low-Rox mix, 400 nM of reverse and forward primers, 100 nM of probe, 2 µl of DNA template and nuclease-free water up to the final volume. The qPCR machine was programmed for an initial denaturation stage at 95$^{o}$C for 5 min, followed by a PCR stage of 45 cycles of 95$^{o}$C for 45 sec and 60$^{o}$C for 1 min. Negative controls were prepared with no template and positive controls with *A. marginale* Jaboticabal strain. PCR efficiency (90%≤ efficiency ≤115%) was determined generating sequence-specific standard curves with 5-fold serial dilutions of DNA from the positive controls. All reactions were loaded in triplicate into 96-well plates (Bioline, Portugal). The threshold was manually set at 0.05 for *A. marginale*. The data generated for each plate was analysed using the Applied Biosystems 7500 Fast 2.0.6 Software (Alfagene, Portugal). Samples with quantification cycle ($C_q$) values above 35 for *A. marginale* were considered negative for the presence of the pathogen.

### *Statistical analysis*

At the time of sample collection, the age of and the sex of 253 animals were recorded. For the age classification, animals were considered weaners if they were between 1 and 2 years old and adults if they were older than 2 years old. Statistical analysis was conducted to determine the effect of *A. marginale* infection on cattle age, sex and

PCV using Microsoft Excel (Microsoft Office Professional Plus 2013) and SPSS Statistical software (Version 23; IBM). Univariate analysis using Chi-square test ($\chi^2$) or Fisher's exact test (Plackett, 1983) and Pearson's correlation (R) were carried out. The level of significance was set at $p < 0.05$. Animals with missing PCV values were excluded in the univariate analysis.

## *Results*

Between April and August 2013, a total of 253 blood samples were collected from bovines of 11 cattle producing villages in Edu LGA that included Bacita, Belle, Bokungi, Fanagun, Fedudangi, Gonandogo, Mokwagi, Ndabata, Ndachewoye, Tshonga and Yelwa. Most of the samples were collected at Ndachewoye 64/253 (25.3%), Fanagun 56/253 (22.1%) and Gonandogo 40/253 (15.8%).

Results of the descriptive statistics have shown that 11.1% (28/253) were weaners, whereas 88.9% (225/253) were adult animals. Regarding the sex of the animals, 45.5 % (115/253) were males and 54.5% (138/253) were females.

To determine the presence of *A. marginale* in the surveyed animals by qPCR, DNA was extracted from 253 blood samples stored on Whatman® FTA® cards. The specific primers and TaqMan® fluorescence-labelled probes that amplify a fragment of the *msp*1b gene of *A. marginale* (Carelli et al., 2007) assured the specificity of the reaction. All the reaction plates were validated by the absence of amplification in the negative controls and by amplification with the positive controls of *A. marginale* Jaboticabal strain. The 5-serial dilution standard curves that were generated allowed to determine the efficiency of each reaction plate automatically with the Applied Biosystems 7500 Fast 2.0.6 Software (Stolovitzky and Cecchi, 1996). The efficiencies of the plates were within the range 90-115%, also validating the reaction. Regarding the detection of pathogens, for *A. marginale*, a total of 192 of the 253 blood samples were considered positive, which corresponds to an overall infection rate of 75.9%. The village Belle presented 20/20 (100%) of the samples infected with *A. marginale*, closely followed by Ndachewoye 59/64 (92.2%), Fedundagi 10/11(90.9%), Bacita 6/7 (85.7%) and Bokungi 10/12 (83.3%). In the villages Gonandogo, Tshonga and Yelwa *A. marginale* was detected in 75% of the collected samples. With a lower infection rate were the villages Ndabata, Mokwagi and Fanagun with 57.1%, 50% and 48.2%,

respectively. The number of positive samples found in each village sampled is described in Figure 2.

When considering the sex classification and the presence of infection, it was observed that the percentage of infected males and females was approximately the same. Eighty-six of the 115 males were positive (74.8%), whilst 104 of the 138 females were found positive (75.4%), the difference not being statistically significant ($p > 0.9$). Looking at the cattle age groups and the detection of infection, the prevalence of *A. marg*inale was significantly higher ($p < 0.05$) in adult cattle 174/225 (78.4%) when compared to weaners 16/28 (57.1%).

The PCV of 233 animals was determined using the micro haematocrit method and grouped into five classes (10-20%; 21-30%, 31-40%; 41-50% and 51-60%). The results of the number of positive samples *per* PCV class are shown in Figure 3. Twenty of the 253 cattle had missing PCV results and were excluded from the haematological analysis. It was observed that 66.7%, 78.8%, 78.2%, 73.9%, and 50% of the animals that presented a PCV between 10-20%, 21-30%, 31-40%, 41-50%, and 51-60% respectively were infected with *A. marginale,* the differences not being significant ($p > 0.8$). A weak negative correlation was observed between the presence of *A. marginale* infection and the values of PCV ($R = -0.026$).

## *Discussion*

Having accurate disease diagnosis within animal populations is a necessary precursor for designing appropriate control strategies. To date, the prevalence of tick-borne pathogens in cattle from Nigeria has been assessed by parasitological examination of Giemsa stained blood smears (Kamani. et al., 2010), and in ticks collected from cattle and dogs by molecular methods, such as conventional PCR (Kamani et al., 2011; Ogo et al., 2012). Although conventional PCR has been shown to be useful to determine the presence of *Babesia* and *Anaplasma* (Figueroa et al., 1993; Martins et al., 2008; Bilgiç et al., 2013; El-Ashker et al., 2015), more recently, qPCR has revealed to be more sensitive than the first for the detection of protozoan parasites in cattle (Criado-Fornelio et al., 2009). For the detection of anaplasmosis, some of the TaqMan® based qPCR methods involve the amplification of species-specific genes such as the major surface proteins (*msp*) genes (Carelli et al., 2007; Bacanelli et al., 2014), and six *msp*

(*msp* 1a, 1b, 2, 3, 4, 5) with the potential to be used in diagnostic assays have been identified on *A. marginale* (Kocan et al., 2003). The *msp*1 gene complex (composed of *msp*1a and *msp*1b) has been shown to be a well-conserved gene in *A. marginale* (de la Fuente et al., 2001; Bowie et al., 2002), whose products act as adhesin during invasion of bovine erythrocytes (McGarey et al., 1994). In the present study, the TaqMan® qPCR used were highly specific, and their sensitivity has been previously evaluated for *A. marginale* (Carelli et al., 2007). Probe-based qPCR methods are more expensive but, when compared to other PCR methods have multiple advantages, including the detection of very low infections, no requirement of post PCR steps, and they are a useful quantitative technique for chemotherapeutic and vaccine evaluation ( Monis et al., 2005; Bacanelli et al., 2014). They are also useful in processing several samples simultaneously for large scale epidemiological studies (Carelli et al., 2007). Our results have shown an overall prevalence of 75.9% for *A. marginale*, being greater than 40% in all the villages studied. This result suggests that the disease in the region of Kwara State is endemic and may have become endemically stable for *A. marginale*. This endemic stability for anaplasmosis was similarly reported in a previous study in Brazil (Bacanelli et al., 2014). Endemic stability is an epidemiological state, in which there is high haemoparasites-infected ticks but clinical disease is absent in the population (Jonsson et al., 2012). *A. marginale* was previously reported as the most prevalent (53.5%) haemoparasite in ticks removed from Nigerian cattle (Reye et al., 2012) and the tick vector *Rhipicephalus* subgenus *Boophilus* is also the most prevalent in cattle in Nigeria (Lorusso et al., 2013). Furthermore, the high prevalence of anaplasmosis reported in this study may also suggest the detection of carrier or chronically infected animals with low parasitaemia. This is important in the epidemiology of anaplasmosis because these previously infected cattle can now serve as an infective reservoir for the disease. The principal source for infection with *Anaplasma* is a persistent carrier state that normally occurs after the animal recovers from the acute phase of the disease (Aiello, 1998; Kocan et al., 2000; Bock et al., 2004). Acting as reservoirs for tick transmission, these animals contribute to the propagation of anaplasmosis to susceptible hosts, thereby increasing the number of newly infected animals. Moreover, carrier animals during a stressful condition or poor nutrition are at risk of succumbing with clinical disease.

The lack of significant difference in prevalence of anaplasmosis between sexes of cattle in this study is supported by similar findings from a past study in Sudan on haemoparasitic infection (Awad et al., 2011). In this study, adult cattle had a significantly higher prevalence ($p$=0.013) of infection (78.4%) compared to weaners (57.1%). This finding is in agreement with past studies in Nigeria where this difference in prevalence between age groups (Kamani. et al., 2010). A negative correlation might be expected between PCV and *A. marginale* infection because anaplasmosis is a haemoparasitic disease characterised by progressive anaemia due to destruction of erythrocytes by phagocytosis. The weak negative correlation observed here was consistent with this expectation but not statistically significant. A reduction in PCV in Nigerian livestock has been shown in infections with tick-borne parasites characterised by anaemia, including in *A. marginale* infection (Obi and Anosa, 1980). This observation is in agreement with another study in Nigeria that reported a reduction in PCV due to haemoparasitic infections (Kamani. et al., 2010). In a similar study carried out in Ghana, the PCV values in cattle studied were significantly higher in non-infected animals compared to those with haemoparasitic infection (Bell-Sakyi et al., 2004).

## *Conclusion*

Ticks and TBDs are considered an emerging global threat both for humans and animals. The results of this study indicate that in the 11 villages of Edu LGA, in Nigeria, where the blood samples were collected *A. marginale* was detected with high prevalence rates. Also, it was confirmed that the qPCR TaqMan® based assay is indeed sensitive and effective for the detection of tick transmitted haemoparasites, revealing more positive samples than previous studies carried out in Nigeria. The confirmation of the presence of *Anaplasma* in Nigeria is of extreme importance as this pathogen has great impact in animal health, influencing animal production. Finally, information about the prevalence of infection in cattle is essential to provide information in order to develop future effective preventive and control strategies.

## Conflict of interest

The authors declare no competing personal or financial interests.

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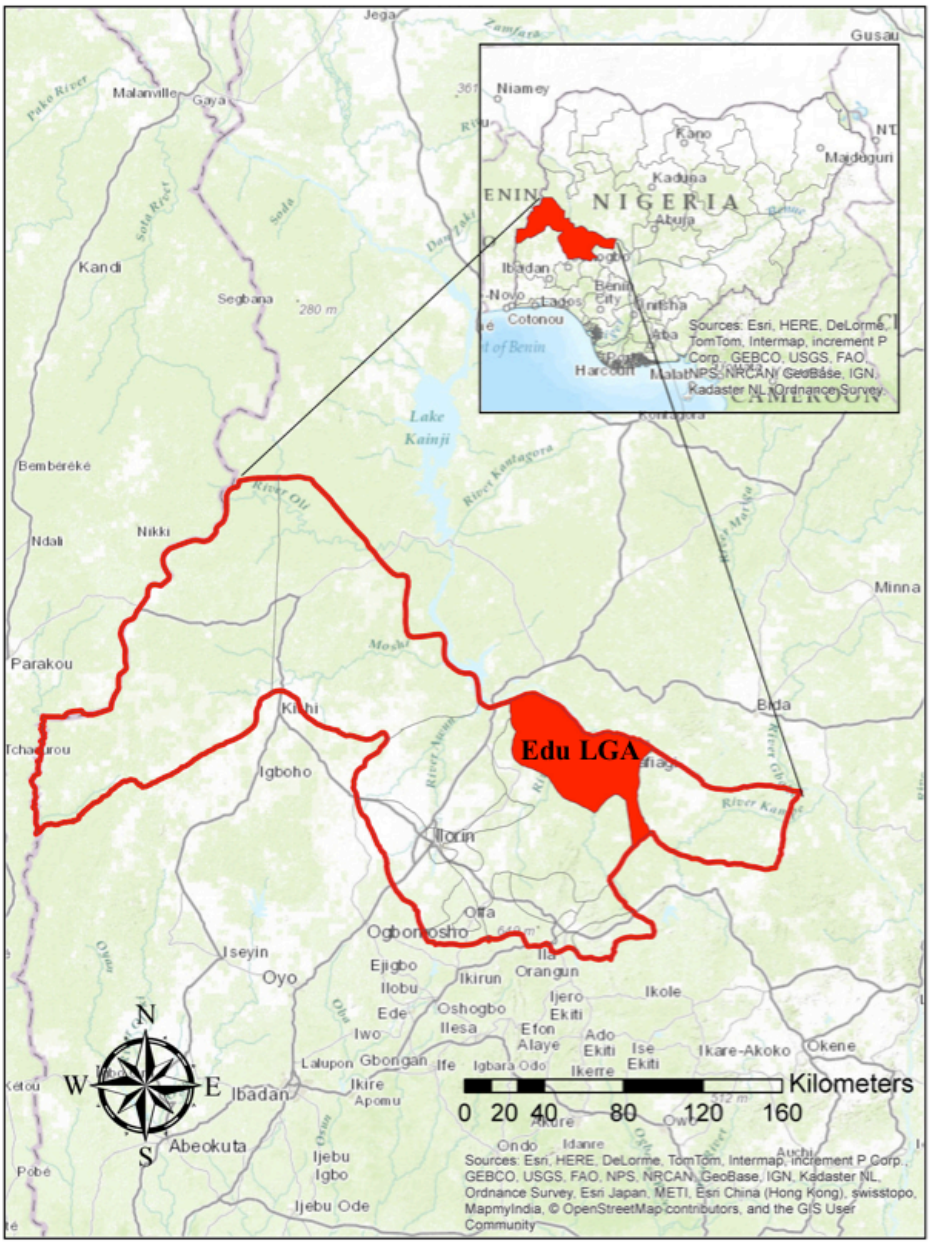
Edu LGA
NIGERIA
Niamey
Kano
Kaduna
Abuja
Ibadan
Cotonou
Harcourt
Maiduguri
Gusau
Kandi
Segbana
Bembéréké
Nikki
Ndali
Parakou
Kilibi
Igboho
Tchaourou
Lake Kainji
Minna
Bida
Ilorin
Offa
Ogbomosho
Iseyin
Oyo
Ejigbo
Ilobu
Ede
Iwo
Oshogbo
Ilesa
Ikirun
Orangun
Ila
Ijero
Ekiti
Efon
Alaye
Ado
Ekiti
Ise
Ekiti
Ikole
Ikare-Akoko
Okene
Ikire
Apomu
Gbongan
Lalupon
Ife
Ibadan
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**Figure 1**: Locations where the cattle blood samples were collected. **(A)** Kwara State showing the location of Edu Local Government Area, Nigeria. **(B)** Location of the 11 cattle producing villages in Edu Local Government Area of Kwara State. Maps were constructed using Arc Map software version 10.2.2.

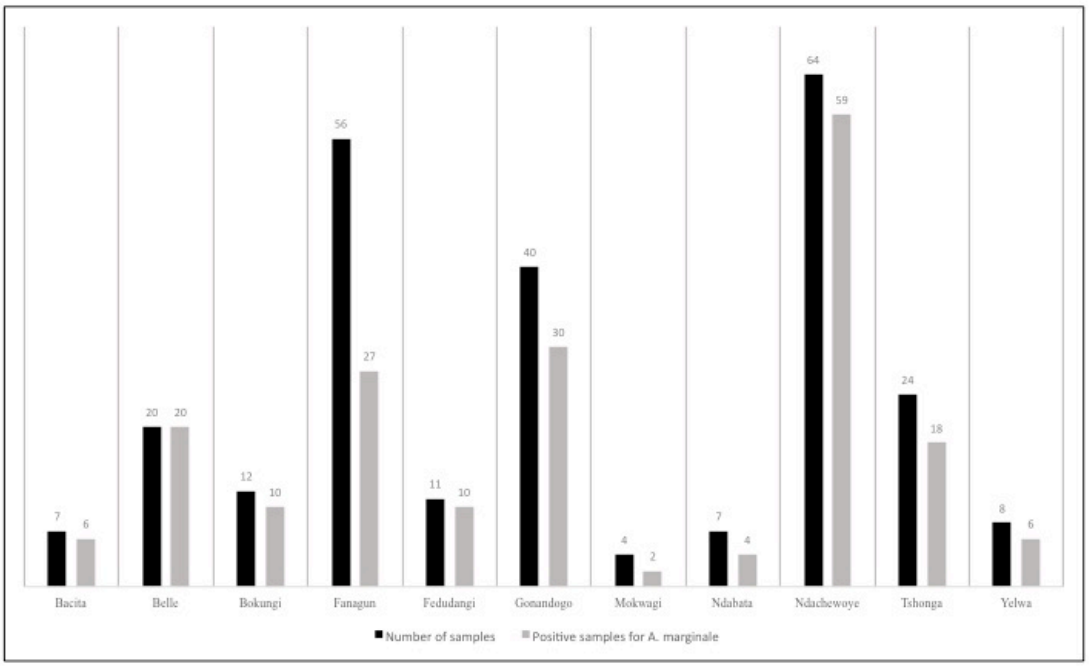


**Figure 2:** Villages of Edu LGA in Kwara State, North-central Nigeria where the blood samples were collected between April and August 2013. Black - Number of samples collected from each village, with a total of 253 samples; Grey - Number of positive samples for *Anaplasma marginale*.

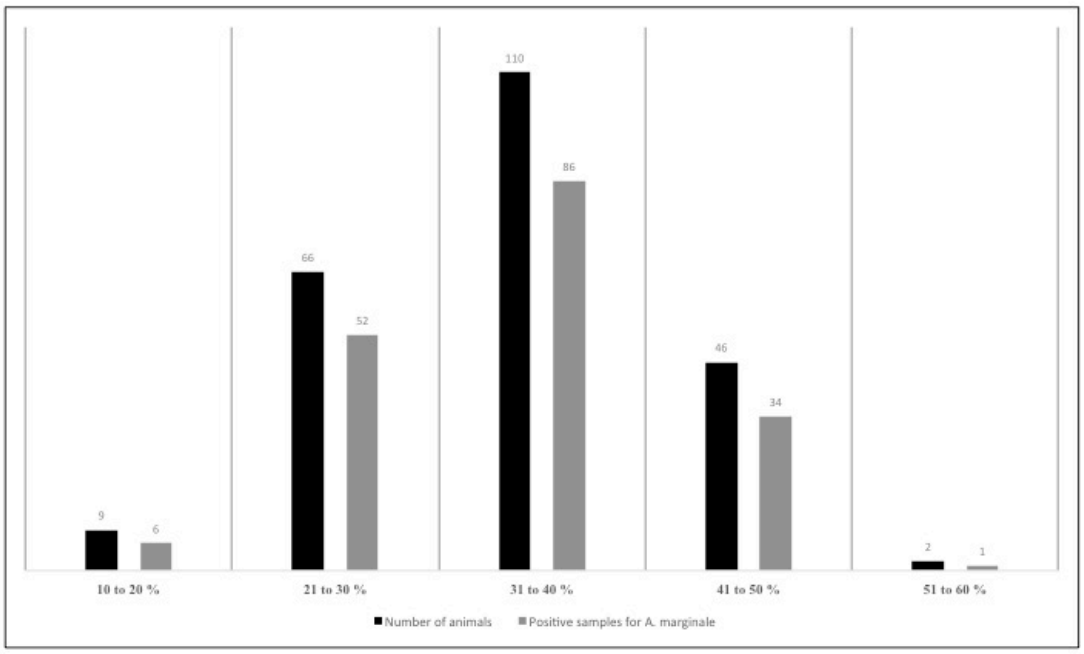


**Figure 3:** Packed cell volume (PCV, %) determined using the micro haematocrit method. Black - Number of animals according to the % of PCV; Grey - Number of positive samples for *Anaplasma marginale*.