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Research article

Prevalence of *Ehrlichia Canis* in Dogs in Makurdi Metropolis Using Methanol Leaf Extract of *Lawsonia inermis*, Giemsa and Romanwsky (Differential Quick) Staining Methods

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ABSTRACT

The important of quality stain for proper diagnosis of haemoparasites cannot be overemphasized in parasitology. Natural stains are safer and more eco-friendly than synthetic ones. Henna leaves methanolic extract solution was optimized and used alongside Giemsia and Romanowsky stains to study prevalence of canine ehrlichiosis in Makurdi. Henna leaves methanol extract solution was optimized, 10% for 45 minutes stain was used independently alongside Giemsia and Romanowsky stains to study prevalence of *Ehrlichia canis* in dogs. One hundred dogs of three different breeds were sampled from four different locations within Makurdi and examined under microscope. The study revealed that henna is not a good stain for haemoparasites since it cannot be picked by the surface glycoprotein of the cell membrane of the parasites. Romanowsky stain revealed 3% prevalence while others recorded zero prevalence. Adult, male and exotic breed of dogs recorded higher prevalence than young, female and other breeds of dogs. The prevalence recorded agreed with the previous reports from western Nigeria but was at variance with reports from other part of the world. Low sensitivity of the method used and the stages of the disease may be possible reasons for low prevalence observed in the study. Henna is not recommended as a good stain for diagnosis of haemoparasites. Breed and age are predisposing factors to the disease, but sex has no significant association with the disease. A more sensitive method and different period of the year should be used for future similar study.

Keywords: *Stain, Ehrlichia, Parasite, Dogs, Prevalence, Diagnosis.*

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INTRODUCTION

A good slide preparation and quality staining is very important for proper identification and diagnosis of haemoparasitic infections through the conventional microscopic examination method. Stains and dyes are frequently used in biology and medicine to highlight structures especially in tissues and cells for studying under different microscopes (Hafiz et al., 2012). Dyes appear to be colours because they absorb some wavelengths of light preferentially, and are obtained from animal and plant sources (Carleton et al., 1976). Until the middle of the nineteenth century, all dyes available to man came from natural sources, mainly vegetables extracts and a few from animal products.

Henna (*Lawsonia inermis*) is a plant which grows wild in abandoned areas (Muhammad and Mustapha, 1994), and is commonly refers to as Lalle in Nigeria major languages with slight difference in pronunciation and is globally known as cosmetic agent used to colour hair, skin and nails (Henna et al., 1998). It produces a red orange dye molecule, Lawsone, which is known as hennotannic acid, that has an affinity for bonding with protein and this has been used to dye skin, fingernails, hair, leather, silk and wool (Singh et al., 2005). It is known that henna stain have been successfully used as a counter stain in Gram staining (Chukwu et al. 2011 and Hafiz et al. 2012) and in histopathological staining (Lizbeth et al. 2018). The following are the chemical constituents that have been isolated from henna; Naphthoquinone derivatives (Lawson), Phenolic derivatives, Coumarins, Xanthones, Tannins, Flavonoids, Aliphatic components, Triterpenes, Sterols, Glucose, Gallic acid, Amino acid, Mannitol, Trace elements and minerals (Tekle et al.,2013). The first human made synthetic organic dye, Mauveine, was discovered by William Henry Perkin in 1856. Many thousands of synthetic dyes have since then been prepared which has quickly replaced the traditional natural plant dyes. They offer a vast range of new colours, and impart better properties upon the dyed materials (Rosenberg, 1971). However, the use of synthetic stains pose a possible threat to the ecosystem hence, the need for exploration of naturally available stain that could substitute the synthetic stains (Lizbeth et al. 2018).

Ehrlichia is a rickettsia bacterium which belongs to the family Ehrlichiaceae. There are several species of Ehrlichia,

but the one that most commonly affects dogs with severe clinical signs is Ehrlichia canis which infects monocytes in the peripheral blood (Harrus et al., 2012). It is naturally transmitted transtadially and intrastadially but not transovarially, by tick Rhipicepalus sanguineous (Sainz et al., 2015). The organism is found in many parts of the world and was first recognized in Algeria in 1935. The species are intracellular cocci that infect different blood cells of various animal species and human (Lauren et al., 2003). Although, it is primarily the pathogen of animals but the zoonotic potential has also been reported (Ettinger and Felman, 2000). The disease is characterized by the presence of intracytoplasmic inclusion bodies (Morulae) in circulating monocytes and lymphocytes which serve as a diagnostic tool (Mylonakis et al., 2003). However, it is difficult to reach a definitive diagnosis based only on clinical and hematological abnormalities as natural infections may be present with a variety of clinical signs that vary from between different geographical regions (Asgarali et al., 2012). Fever (occasionally hyperthermia in profoundly pancytopenia dogs), depression/lethargy, anorexia, lymphadenomegaly, splenomegaly, mucosal pallor, ocular abnormalities and bleeding tendency are typical clinical manifestations in the naturally-occurring disease (Sainz et al., 2015). The correlation of the prevalence of disease with various risk factors such as age, sex, breed of host and season have been widely documented earlier from other parts of the country and the world (Harrus et al. 1997; Lakshmanan et al. 2006; Singh et al. 2011; Silva et al 2012) but not from the study area using strictly thin blood smears with different stains. The present study was aimed at investigating the staining quality of henna leaf extract solution in diagnosis of haemoparasites and to compare it with commonly used stains in checking prevalence of canine ehrlichiosis and access the association of some risk factors (breed, sex, age, management system) to the occurrence of the disease in dogs.

MATERIALS AND METHODS

Study Area: The study was conducted in Makurdi, the capital of Benue State. It is located in the middle belt, North Central Nigeria and it lies between longitude 8°35'E and 8°41'E and latitude 7°45'N and 9°52'N. It has a population of approximately 297,398 and it is placed at 106.4m above sea level (Manyi *et al*., 2014; NMA, 2011). Makurdi climatic condition is influenced by two air masses: the warm, moist southwest air mass, and the warm, dry north-east air mass. The south-west air mass is a rain-bearing wind that brings about rainfall from the months of May to October. The dry north-east air mass blows over the region from November to April, thereby bringing about seasonal drought. The mean annual rainfall in Makurdi is about 1,290 mm (Akintola, 1986). Temperature in Makurdi is however, generally high throughout the year, with February and March as the hottest months. Temperature varies from a daily maximum of 40^0C and minimum of 22.5^0C (Temi and Tor, 2006).

Preparation of Henna Methanol Extract: Fresh leaves of henna plant were harvested from henna tree in Makurdi environment and immediately taken to the laboratory. The leaves were air dried exclusively under the shed in the Department of Veterinary Physiology laboratory of College of Veterinary Medicine, University of Agriculture, Makurdi for period of two weeks. The dried henna leaves were grounded with the aid of pestle and mortal and the powder weighed 135g. The powder was then used for the methanol henna extraction as described by Qing-Wen *et al*. (2018). The extract was filtered through filter paper and concentrated in water bath at 40ºc until the weight remained constant after complete evaporation of methanol. The end product of the extract weighed 31.2g, which was stored in air tight plastic container and kept in the refrigerator at 4ºc until used.

Henna Optimization: The Henna methanol extract was diluted with physiological buffer saline to different concentrations for staining blood smears to determine the clearest (best) staining concentration as described by Ogbaje *et al.* (2019a). Various concentrations of henna stain (2%, 3%, 5%, 10% and 15%) were used to stain *Trypanosoma brucei* infected blood smears and allowed to stay for different time intervals before examinations under light microscope. The concentration and time with best quality (clearer) was then selected and used alongside with Giemsa and Diff-Quik stain (Romanowsky) (as controls) to stain independently *Trypanosoma brucei* infected blood smears. These three stains (Henna concentration and timing with best quality, the conventional Giemsa and Romanowsky stains) were later used independently to study the prevalence of *Ehlrichia canis* in dogs in the study area.

Animals for the study: There are three major types of dog keeping systems in Makurdi; the free roaming, semi-confined and confined management systems (Amuta, *et al.*, 2010). The free roaming system is the most commonly practiced, followed by semi confined and few confined system were encountered during this study The dog breeds observed during the study were Mongrel (Local), Mixed (Crossed) and Foreign (Exotic) breeds. The predominant breed was foreign (exotic), closely followed by the mongrel (Local) and very few mixed (crossed) breeds.

Sample Collection and Analysis: One hundred (100) dogs (51 males and 49 females) were randomly sampled from four locations (University of Agriculture Makurdi Veterinary Teaching Hospital Annex, residential houses in North Bank, Owners occupier in South Bank, and Ujam village) in Makurdi. The dogs were restrained in either sternal recumbences or dog siting position, and the collection sites cleaned with 70% alcohol to establish aseptic condition. Blood were collected from the forelimbs (cephalic vein), with 21-gauge needle and immediately transferred into EDTA-coated sample test tubes and rock gently to prevent clotting. After collection pressure was applied at the site of collection to prevent haemorrhage. The samples were transported in ice pack to the University of Agriculture Veterinary Teaching Hospital Parasitology Laboratory for processing. On arrival at the laboratory the samples were analyzed via thin blood smear, stained and examined under light microscopic for morulae of *Ehrlichia canis.* Packed cell volume (PCV) of each animal was determined as according to Cole, (1986). Also the Plasma Protein was determined as described by Kerr, (1989).

Statistical Analysis

All the data generated were subjected to Analysis of Variance (ANOVA) using SPSS 20 Software (SPSS Inc. IL, USA). Significant different between the stains were checked/separated using Duncan Multiple Range Test, P value ≤ 0.05 was considered significant

RESULTS

The optimization of henna stain revealed 10% concentration for 45 minutes to be the best (clearer).

Prevalence based on stains used: Out of the hundred (100) dogs sampled, 3 (3%) dogs were found positive with Romanowsky (Diff-Quik^{R}) stain, Henna and Giemsa stains recorded zero each. Of the three positive cases, two were males (1.02%) and one was female (0.49%) as presented in table 1.

Prevalence based on sample collection site: All the positive cases were obtained from samples of dogs presented at the Veterinary Teaching Hospital Annex, Federal University of Agriculture, Makurdi. Samples from the other three locations were all found negative to the morulae of the parasite (*Ehrlichia canis*). The numbers of male and female dogs sampled from each location were: VTH, 43: 39, North Bank 3: 3, Ujam 1: 4 and Owner Occupier 4: 3 respectively. Two of the positive dogs were males and one was female as shown in table 2.

Prevalence Based on Age: Dogs (six months and below) were considered puppies. Seventy-two adult dogs and twenty eight puppies were sampled. All the three positive dogs were adult, none of the puppy was found positive of *Ehrlichia canis* (table 3).

Prevalence based on Breed: Although more (50) of the Exotic breed were sampled which revealed 2 (4%) positive cases, this was closely followed (40) by the local breed that recorded 1(2.5%) confirmed case. The cross/mixed breed (10) recorded zero positive as presented in table 4.

DISCUSSION

The important of Stain in microscopic diagnosis of haemoparasites cannot be over emphasized. However, most of the stains that have been in used where synthetic stains which pose serious threat to the ecosystem (Lizbeth *et al*., 2018) hence, there is need to explore for better naturally available ones to substitute the synthetic ones. However, this study was conducted to explore Henna leaf extract as a natural stain for microscopic diagnosis of haemoparasites. The study revealed that Henna leaf extract is not a good stain for haemoparasites diagnosis since the stain cannot be picks by the surface glycoprotein of the cell membrane of the parasite. However, it has been reported that henna stain have been successfully used as a counter stain in Gram staining (Chukwu *et al*., 2011; Hafiz *et* al., 2012) and histopathological staining (Lizbeth *et al*., 2018) when oxidized and used with a mordant which improved the affinity of the dye to the tissue.

Table 1:
Prevalence of *Ehrlichia canis* based on stain type and animal sex.

Stain Type/ Total Samples examined (N)	Total No of male Examined	Total no of female Examined	Total No of male positive/ Percentage positive	Total No of female positive/ Percentage positive
Henna	51	49	0(0%)	0(0%)
Giemsa	51	49	0(0%)	0(%)
Romanowsky (Diff-Quik)	51	49	2(1.02%)	1(0.49%)

Table 2:
Prevalence of Ehrlichia canis in Makurdi based on sample collection sites and sex

Location	Total Nos Dogs screened	Males	Females	Infected males (%)	Infected females (%)	Total dogs infected (%)
VTH	82	43	39	2 (1.05%)	1 (0.48%)	3 (3.66%)
North bank	6	3	3	0	0	0
Ujam village	5	1	4	0	0	0
Owners Occupier	7	4	3	0	0	0
Total	**100**	**51**	**49**	**2**	**1**	**3 (3%)**

Table 3
Prevalence of *Ehrlichia canis* in Makurdi based on age.

Age of dogs screened	Total number of dogs screened	Number of dogs infected	Prevalence (%)
Adult	72	3	4.17
Puppy	28	0	0
Total	**100**	**3**	**3**

Table 4
Prevalence of *Ehrlichia canis* in Makurdi based on Breed

Breed of dogs screened	Total number of dogs screened	No of dogs infected	Prevalence (%)
Exotic	50	2	4
Local	40	1	2.5
Cross	10	0	0
Total	**100**	**3**	**3**

The prevalence of 3% recorded in this study (which was only detected in Romanowsky stain) is in agreement with Olukayode *et al*. (2018), Subhash *et al*. (2013), Milanjeet *et al*. (2014) and Parmar *et al*. (2012) who reported 1.5%, 1.72%, 2.34% and 2.5% respectively from western part of Nigeria. Authors observed in the course of the research that slides prepared using Romanowsky stain were better/or clearer than those stained with Henna and Gimesia, which maybe the likely hold of zero prevalence recorded in those slides stained with the other two stains. Conversely, the 3% prevalence was lower than what was reported by Paula *et al.* (2015), Albernaz *et al*. (2007) and *Moreira et al*. (2003) who reported 12.4%, 13.89% and 15.97% respectively from South America (Brazil) and Manasa *et al.* (2017) who also reported 14.28% from India. The variation in the prevalence from different regions may be associated with climatic conditions which may have direct effect on the vectors (ticks) and also the period of sampling (high or low vector population). Differences in climatic conditions have been reported to be important factors that influence the population dynamics of ticks in a particular region thereby resulting in a variable prevalence pattern of canine ehrlichiosis (Costa *et al*., 2007). The stages of the disease during sampling (Acute, Subclinical or Chronic stage) also have great effect on the diagnosis of the disease. The low sensitivity of conventional thin blood smear microscopic examination method of diagnosis of this disease especially during subclinical stage of the disease may also attribute to the low prevalence recorded in this study as earlier reported by Woody and Hoskins, (1991) and Harrus and Waner, (2011). It is well known that in the subclinical stage of the disease, the pathogen is sequestered within the spleen and hence not detected in the blood (Waner, 2008). Other limitation for the use of microscopic method of diagnosis is that it is difficult to differentiate morulae from the inclusions present during severe bacterial infections (like Dohle bodies), inflammation, viral infections (like canine distemper) and severe tissue damage which may result in false positive (Schalm 2000). Therefore, molecular diagnosis, based on sensitive and specific PCR assays will be helpful especially in the early diagnosis of the disease along with the identification of the infecting species, which may eventually help in taxonomic classification (Iqbal *et al*., 1994).

The relatively high prevalence in the adult dogs compared to the young dogs varied with previous reports of higher incidence of haemoparasites in young than adult dogs (Ezekoli *et al*., 1983*;* Abdullahi *et al*., 1990; Samaradni *et al*., 2005; Lakshmanan *et al*., 2006). The study revealed that age can be considered as one of the risk factor for the disease.

It was observed in the study that more male dogs were infected than the female dogs but with statistical insignificant different. This was also in agreement with the earlier reports by other researchers that have used either the conventional microscopy or serology and molecular methods that sex is not a risk factor for the disease (Lakshmanan *et al.* 2006; Kumar *et al*. 2009; Singh *et al.* 2011; Silva *et al*. 2012).

The high prevalence of the disease in the Exotic breed of dogs in the study area is in agreement with the earlier reports that foreign breed of dogs are more susceptible to canine ehrlichiosis than the indigenous/or local breeds of dogs (Huxsoll *et al.* 1972; Heerden, 1982; Lakshmanan *et al*. 2006). This may be due to low level of acquired immunity either from the bitch (maternal) or previous exposure by the local breed of dogs which may be lacking in the exotic breed and this may likely result to defective cell-mediated immune response as seen in the exotic breed.

In conclusion, Henna leaf extract is not a good stain for diagnosis of haemoparasites due to its inability to stain the surface glycoprotein parasites and low (3%) prevalence of the organism was recorded in the study area. Romanowsky (Diff-Quik) stain proves to be better stain than the other two stains in the diagnosis of canine ehrlichiosis. The study has shown that age and breed are risk factor for the disease but sex is not a risk factor.

The authors recommend that prevalence study of canine ehrlichiosis in the study area should be repeated using serological and if possible molecular methods to ascertain the true status of the disease in Makurdi and its environs. Also that better stains such as Romanowsky (Diff-Quik) stain should always be made available and use in our laboratories for screening animals for haemoparasites. Finally, further research work should be conducted to explore other uses of henna leaf extracts as a biological stain to enhance the laboratory diagnosis of haemoparasites in developing countries like Nigeria.

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