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Research article

Molecular Diagnosis of Bovine Anaplasmosis in District Lahore

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Abstract

Bovine anaplasmosis is a tick borne rickettsial disease wide spread in tropical and subtropical areas. A comparative study was conducted to check the prevalence of bovine anaplasmosis in different age groups of cattle and buffaloes. A total of 160 samples were collected (80 from each) during May-August. Screening was done by blood smears, stained by Giemsa'wright staining technique and later the blood samples from the same animals were also processed by PCR. On the basis of microscopic examination, overall 11.25% (18/160) disease prevalence was recorded. On the basis of polymerase chain reaction (PCR) prevalence of *Anaplasma marginale* 25.6% (41/160) was recorded, showing the presence of carrier animals in District Lahore. The polymerase chain reaction showed that the prevalence of bovine anaplasmosis is more in cattle 32.5% (26/80) than in buffalo18.75 % (15\80). The results have demonstrated the high efficacy of polymerase chain reaction assay as compare to microscopic examination.

Key words: *Anaplasmamarginale*, PCR, Buffalo, Cattle, Prevalence.

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Introduction

Livestock is an important sector of agriculture in Pakistan, which accounts for 51.8% of agriculture value added and about 11.3% of the GDP during 2008-2009. The population of Buffalo is 29.9 million and of cattle is 33.9 million in Pakistan. Production of cattle milk is 27,028, million tons, meat 2,515 million tons and hides 6,260 million tons [1]. According to the 1997 FAO report, the buffalo are recognized as "Black Gold of Asia" [2]. Research on water buffaloes has been much neglected in comparison to research work on cattle [3]. Buffaloes are the principal source of milk and meat production in Pakistan while cattle comprise a second source. In Pakistan, parasitic diseases, including tick born are the major obstacle in health and performance of animals.

Bovine anaplasmosis is usually caused by *Anaplasma marginale* [3]-[6]. These obligate intracellular organisms replicate in membrane-bound parasitophorous vacuoles in bovine erythrocytes. Both cattle and ticks become persistently infected with *A. marginale* and thus serve as reservoirs of infection [7]. The disease is characterized by fever, severe anemia, jaundice, brownish urine, loss of appetite, dullness or depression, rapid deterioration of physical condition, muscular tremors, constipation, yellowing of mucous membrane and labored breathing [8], decreased milk yield, jaundice, abortion and sometimes death [9].

Diagnosis of bovine anaplasmosis can be made by finding *A. marginale* in Giemsa-stained blood smears from clinically infected animals, during the acute phase of the disease. It is not reliable for detecting pre-symptomatic or carrier animals. In these instances, the infection is generally diagnosed by serologic demonstration of antibodies with confirmation by molecular detection methods. Polymerase chain reaction–based methods have been developed that are capable of detecting low levels of infection. In the present study, prevalence of bovine anaplasmosis was studied and it was aimed to check the prevalence of Bovine anaplasmosis in cattle and buffalo and to check the efficacy of diagnostic methods used.

Materials and Methods

Collection of blood samples

A total of 160 blood samples were collected from cattle and buffaloes, randomly from eight villages, during the month of May, June, July, August of 2010 in and around District Lahore. 80 samples were collected from cattle and 80 were collected from buffaloes and these samples were further

categorized into two age groups that is 40 samples were collected from calves of 1 month to 6 month of age and 40 samples were collected from calves of 7 month to 12 month of age of each species.

Microscopic examination

Screening was done by blood smears, stained by Giemsa'wright staining technique and later the blood samples from the same animals were also processed by PCR. Blood samples were collected from jugular vein of cattle and buffaloes. 3ml of blood was drawn in EDTA coated vacationers from each cattle and buffalo. Screening of 160 animals in District Lahore was done by blood smears. Dried smears were stained by Giemsa's staining technique described by [10]. The dried blood film was flooded with methyl alcohol for 10 minutes. The slides were washed gently with tap water to remove alcohol. Fixed slides were stained in working dilutions of Giemsa's stain (1:10) for 30 minutes. The slides were washed with tap water and air dried. A drop of cedar wood oil was placed on the smear and slide was examined under oil immersion lens of microscope for the presence of *anaplasma* and morphology of red blood cells. The slides were examined in the laboratory and the parasitic identification was done with the help of keys mentioned in the book titled helminths, arthropods and protozoa of Domestic animals [10].

PCR analysis

After screening, DNA was extracted from blood obtained from animals by using DNA extraction kit (Gentra, USA). PCR was performed and analysis of extracted DNA was made by agarose gel electrophoresis. In agarose gel electrophoresis based analysis, 0.7% agarose gel was used. For the identification of *anaplasma* and *anaplasma marginale* two set of primer specific to amplify *anaplasma genus* and *anaplasma marginale* were used.

Forward (M60313 AGAGTTGATCCTGGCTCAG, 120) Reverse (M60313 AGCACTCATCGTTTACAGCG, 781-762) forward primer for *anaplasma marginale (*AM100 CAGAGCATTGACGCACTACC, 337-356 bp) reverse (AM101 TTCCAGACCTTCCCTAACTA, 582-563bp), were described by (11) (Table 1).

Amplification was done with the help of thermal cycler set for 30 cycles. Analysis of amplified product was made by gel electrophoresis. The amplified product was separated by electrophoresis on a 1% agarose gel.

Table 1: Oligonucleotide Primers used to amplify 30-KDA and SSU RRNA gene sequence of *Anaplasmamarginale* (Primer sequence A position B characteristic)

Primer	Primer sequence	Target position	Target region	size
Primer Set A: *Anaplasma marginale* specific				
AM100	CAGAGCATTGACGCACTACC	337-356	SSU rRNA gene	246
AM101	TTCCAGACCTTCCCTAACTA	582-563	SSU rRNA gene	
Primer Set B: Anaplasma specific				
M60313	AGAGTTGATCCTGGCTCAG	1-20	SSU rRNA gene	781
M60313	AGCACTCATCGTTTACAGCG	781-762	SSU rRNA gene	

Results

Blood smear examination

Microscopic examination revealed maximum prevalence in the month of June 22.5% (9/40), followed by May and July 10% (4/40) while minimum prevalence 2.5% (1/40) was recorded in August. The blood smears showed maximum prevalence in cattle of age 7 months to 12 months of age that is 20% (8/40) than animals of age 1 month to 6 month of age 10% (4/40) (Figure 1)

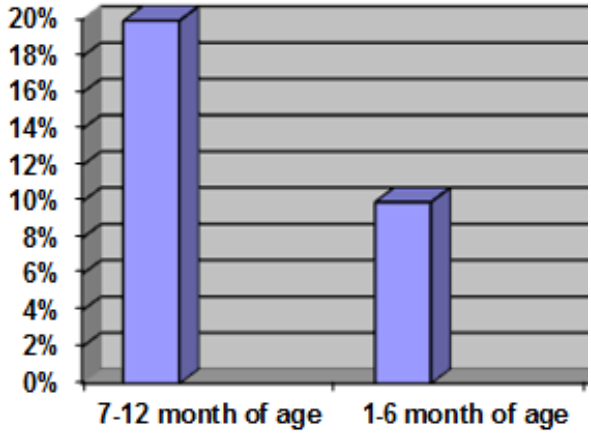

Fig 1. Age wise prevalence in Cattle

while prevalence in buffaloes of age 7 months to 12 months of age that is 10% (4/40) than animals of age 1 month to 6 month of age 5% (2/40) (Figure 2). The blood smears showed that the prevalence of bovine anaplasmosis is more in cattle 15% (12/80) than buffalo 7.5% (6/80) (Figure 3).

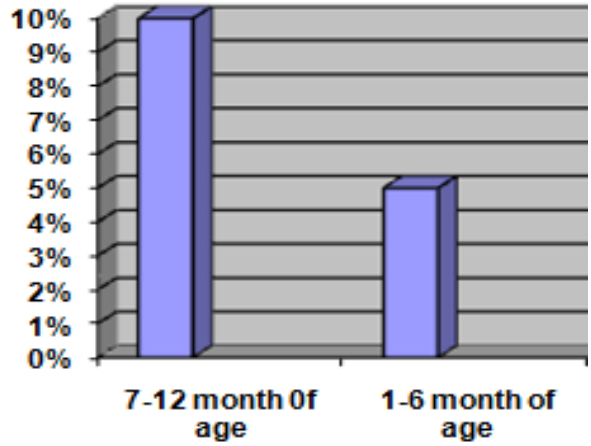

Fig 2. Age wise prevalence in Buffaloes

PCR Evaluation

Primer set M60313/M 60313 and AM100/ AM101 was used in the PCR performed on the blood samples taken from reference population. The 781-bp fragment was generated in all samples tested with M60313/M60313 (Figure 4). For primer set A the expected 246-bp fragment of DNA was amplified in positive samples (Figure 5). The specificity of the amplified DNA fragment was confirmed by positive control samples.

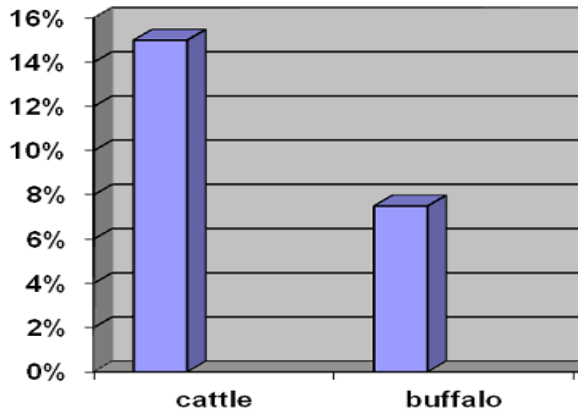

Fig 3. Species wise prevalence based on blood smear examination

The overall prevalence 25.6% (41/160) was recorded for bovine anaplasmosis, during summer season on the basis of PCR. The maximum prevalence was recorded in the month of June 45% (18/40), followed by May 25% (10/40), followed by July 17.5% [7/40], followed by August 15% (6/40).

The polymerase chain reaction showed maximum prevalence in cattle of age 7 months to 12 months of age that was 45% (18/40) than animals of age 1 month to 6 month of age 20% (8/40). The polymerase chain reaction showed maximum prevalence in buffaloes of age 7 months to 12 months of age that was 27.5% (11/40) than animals of age 1 month to 6 month of age 10% (4/40). The polymerase chain reaction showed that the prevalence of bovine anaplasmosis was more in cattle 32.5% (26/80) than buffalo 18.75 % (15/80) (Fig. 6).

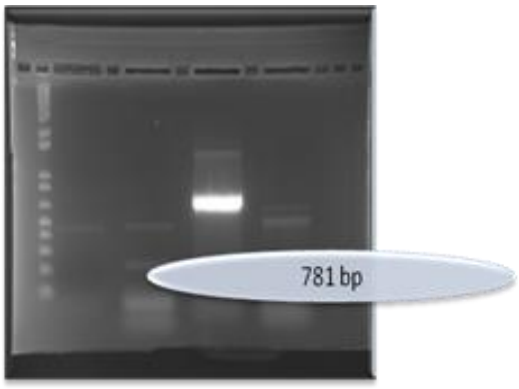

Fig 4. Amplified DNA of *Anaplasma*

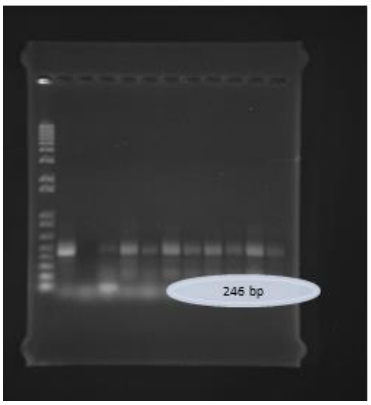

Fig 5. PCR product of Anaplasma marginale

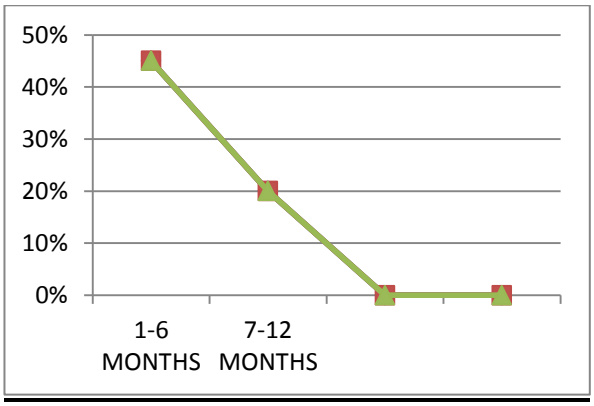

Fig 6. Age wise prevalence in cattle on the basis of PCR

Comparisons of blood smear examination and PCR

The efficacy of microscopic examination revealed during present study was 11.25% (18/160), while efficacy of PCR was recorded 25.6% (41/160).

It was also recorded that the sample positive for blood smears were also positive by PCR, while out of 41 samples, 20 samples found positive by PCR but not detected by microscopic examination. So PCR show high sensitivity of PCR test as compare to microscopic examination (Fig. 7).

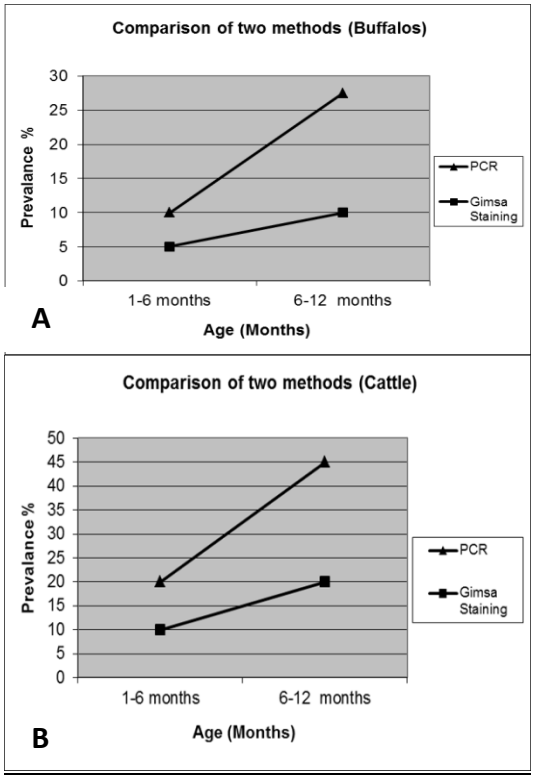

Fig 7. Comparison of blood smear and PCR methods in buffalo (A) and cattle (B).

Discussion

During this study, the disease manifestations observed clinically including pyrexia, anemia, and brownish urine, and jaundice, loss of appetite, constipation, dullness, depression, pale mucous membrane and difficult breath in young animals. These signs were observed during in all four months of summer season (May, June, July, and August), similar findings were reported by Bram *et al*. [8]. Alderink and Diertrich [9] reported *Anaplasma marginale* infection with infecting red blood cells, severe anemia, fever, constipation , weakness, anorexia, jaundice, while Rajput *et al.* [11] reported fever, anemia. Brownish urine, yellowing of mucous membrane, labored breathing, jaundice, depression, dullness. De la Fuente *et al*. [12] observed anemia, icterus, without haemoglobinemia and haemoglobinuria, results from massive phagocytosis of infected erythrocytes by the bovine

reticuloendothelial system. Kocan *et al.* [7] observed the same signs and fever, weight loss, lethargy. Palmer *et al*. [13] observed severe anemia, weight loss, and death. Kocan *et al.* [7] reported that *Anaplasma marginale* invade erythrocytes, which are later removed from the circulation by reticuloendothelial cells, resulting in varying degree of extravascular hemolysis and anemia. Almost similar clinical findings were observed in the present study the most marked clinical signs of bovine anaplasmosis were anemia and jaundice.

Diagnosis of bovine anaplasmosis can be made by demonstration of *A. marginale* on stained blood smears from clinically infected animals during the acute phase of the disease, but it is not reliable for detecting infection in pre-symptomatic or carrier animals. Different molecular biological techniques such as polymerase chain reaction assay has been used in recent years and has been used for the detection and identification of many parasites. Several studies showed that polymerase chain reaction (PCR) assay is more specific and sensitive than conventional techniques in diagnosing the carrier animals [14]. In this study, positivity rate by PCR 25.6% (41/160) was higher than Microscopic examination 11.25 % (18/160). The primers sets used in present study are already described [15].

In this study, for *Anaplasma marginale*, the 246-bp fragment was generated in all samples tested with AM100/AM101. For Anaplasma specific, primer set M60313/M60313 the expected 781-bp fragment of DNA was amplified. Polymerase chain reaction (PCR) is rapid method for diagnosis of infection in environment as well as in food samples. In PCR species specific DNA regions and specie traits of pathogenicity is targeted.

One of the most important animals of Asia, buffaloes on which research rate is very low especially in Pakistan. Buffaloes are able to tolerate the hot climate so these are also susceptible to the parasitic infections but the incidence and the severity of the infection in them is less than that of cattle's. Buffaloes are also susceptible to Bovine anaplasmosis but the severity of disease is less than the cattle. *Anaplasma marginale* is transmitted by the at least twenty tick species [16]. The cattle and buffalo breeds of Pakistan are naturally resistant to the ticks. The present study results showed that tick carrying cattle had higher percentage 32.5 % (26/80) of prevalence of parasite than buffalo 18.75% (15/80) as shown by the PCR. The reason is, main host of bovine anaplasmosis is cattle [17] while the buffaloes are also susceptible to infection and the pathogenic organism but the effects on buffaloes are less severe than on cattle in the same environment. Water buffaloes are generally considered as healthier animal in comparison to cattle [3]. Simmuza *et al*. [18] conducted a study and found 11.8% prevalence of *Anaplasma marginale* infection in Zambias, and Rajput *et al*. [11] found 22% prevalence of *Anaplasma marginale* in Hyderabad District Pakistan, while in the present study it was 25.6%. The reason for these results may be the climatic difference and ticks infestation intensity in that particular area. In this study the overall results showed that PCR showed significantly higher efficacy (25.6%) of detection of anaplasma sp. Compared to (11.25%) microscopic observations of stained blood smears and allowed the pathogenic and non-pathogenic specie discrimination because anaplasma discrimination, which cannot be accomplished by traditional diagnosis by microscopic examination. Similarly Rajput et *al*. [11] conducted the comparative study of anaplasma parasite in tick carrying buffalo and cattle and concluded that cattle and buffalo both are susceptible to *anaplasma* parasite infection but cattle are more susceptible than buffalo, male and female of cattle are equally more susceptible than either sex of cattle. Present study results are similar to Rajput *et al*. [11].

Cattle of all ages are susceptible to infection with *A. marginale* (A review of Bovine anaplasmosis Tran boundary and Emerging Diseases, Volume 58). Molad *et al*. [15] also used the PCR for the molecular detection of *Anaplasma marginale* where 70 samples out of 90 samples were found positive. This happens because different age groups were selected.

Yamada *et al*. [19] used the PCR for the detection of blood parasites in adult cattle in japan where 34 out of 71 samples (47%) were positive for *Anaplasma marginale*. Carelli *et al*. [20] used the PCR for the diagnosis of *Anaplasma marginale* in adult cattle and by using real time PCR, 39 bovine samples out of 51 (76%) were found positive. So the difference between the present studies is because of species difference, climate and age groups.

The results showed overall efficacy of PCR at 25.6% while for the blood smear examination it was 11.25%. These observations were in accordance with Rajput *et al*. [11], Molad *et al.* [15], and Carelli *et al*. [20]. Similar findings were recorded by Molad *et al*. [12]. In the present study the overall prevalence

25.6% (41/160) was recorded for Bovine anaplasmosis, during summer season on the basis of PCR. The maximum prevalence was recorded in the month of June 45% (18/40), followed by May 25% (10/40), followed by July 17.5% (7/40), followed by August 15% (6/40). The blood smears showed overall prevalence 11.25% and maximum prevalence in the month of June 22.5% (9/40), followed by May and July 10% (4/40) and minimum prevalence 2.5% (1/40) was recorded in August, difference among prevalence of months is may be due to tick burden in addition with the environmental factors. The reason of higher incidence in District Lahore may be due to the fact that brick soiling is present in animals.

It is concluded that cattle and buffaloes are susceptible to *anaplasma* infection but cattle are more susceptible than buffaloes and PCR is more specific and sensitive assay than microscopic examination.

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