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Outbreaks of Anaplasmosis in Dairy Cattle in Punjab, India

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ABSTRACT

Two severe outbreaks of bovine anapalsmosis caused by *Anaplasma marginale* were recorded in two districts (Ferozepur and Patiala) of Punjab state in the year 2016. Mortality of animals was recorded in two dairy cattle herd comprising of a total of 260 animals in Ferozepur (n=218) and Patiala (n=42) districts. There was history of purchase of animals at one farm. Out of 260 cattle at risk, 40 were critically ill and 9 died of disease with morbidity, mortality and case fatality rate of 15.38 %, 3.46 % and 22.50 % respectively. Fifteen blood samples were collected from diseased (n=11) and healthy animals (n=4) for haematological analysis, parasitological and polymerase chain reaction (PCR) based diagnosis. Clinical signs in infected animals included high temperature, icterus, anemia, anorexia and decreased milk production. Necropsy findings revealed splenomegaly and severe jaundice. Mild tick infestation was observed at both the farms. Ticks collected from both the outbreaks were identified as *Rhipicephaus (Boophilus) microplus*. Thin blood smears from infected animals were found positive for *Anaplama marginale* organisms & disease was further confirmed by molecular approach (PCR). Affected animals were successfully treated with tetracycline, haematinics and antipyretics. PCR was found to be more sensitive in detecting the disease especially in latent infections. Animal owners were advised to follow quarantine measures before mixing new animals in a herd and strategic acaricidal treatment for effective tick control.

Keywords: *Anaplasma marginale*, Cattle, Outbreaks, Parasitological diagnosis, Polymerase Chain Reaction (PCR).

Anaplasma marginale is an intra-erythrocytic rickettsial organism responsible for severe disease in bovines (Dumler *et al*., 2001). Disease is transmitted biologically by infected ticks or mechanically by biting flies or blood-contaminated fomites and is very common in Indian sub-continent among bovines especially cattle. Though an exact estimate of economic losses due to anaplasmosis in India have not been documented, but an estimated annual loss due to anaplasmosis in the US alone amounts to $100 million (McCallon, 1973). Major clinical symptoms of the disease include fever, anaemia, icterus, weakness, anorexia, dehydration, depression, laboured breathing, abortion and often death (Richey and Palmer, 1990).

Anaplasmosis is traditionally diagnosed by microscopic examination of thin blood smears mainly in acute form of infection. The sensitivity of this method is 10^6 infected erythrocytes per ml of blood (Gale *et al*., 1996). Due to the low parasitemia in carrier cattle, this method is not recommended for the characterization of persistently infected cattle (Carelli *et al.*, 2007). Serological tests may be sensitive or specific for antibody detection (OIE, 2008) but may not be able to differentiate between the current and previous infection.

To know the presence of the active infection, specific and sensitive polymerase chain reaction was developed and applied to detect *A. marginale* DNA from blood of infected animals and ticks (Bekker *et al.,* 2002; Carelli *et al.,* 2007; Ogo *et al.,* 2012), which seems to be more practical for detection of the disease. The present research paper reports the confirmatory diagnosis of two severe

outbreaks of anaplasmosis in dairy cattle on the basis of conventional microscopy and polymerase chain reaction (PCR) targeting the msp 1β gene of *A. marginale*.

MATERIALS AND METHODS

This department received requests from local veterinarians/farmers regarding mortality of animals (cattle) with symptoms of high fever, anaemia and icterus in Ferozepur and Patiala districts of Punjab state. There were two dairy cattle herds comprising of total 260 cattle in Ferozepur (n=218) and Patiala (n=42) where mortalities were recorded. Animals of both the farms were stall fed. Deworming and routine vaccination practices were followed at farms.

Fifteen blood samples were collected from diseased (n=11; 8 adults & 3 calves) and healthy animals (n=4, all adults) for haematological analysis, parasitological and polymerase chain reaction (PCR) based diagnosis. Out of 11 diseased animals, 6 were exhibiting severe jaundice and fever, whereas remaining (n=5) were showing symptom of high fever as only clinical finding. Approximately five ml of blood sample from jugular vein was collected in EDTA coated and plain vacutainers from each animal. Thin blood smears of each blood sample were prepared and examined for haemoparasites under oil immersion (100X) after staining with Leishman stain. Haematological parameters *viz.,* haemoglobin (Hb, g/dL), total leucocytes count (TLC, (10^3cells/ µL) and differential leukocyte count (DLC %) were evaluated.

There was history of purchase of animals at one farm in district Ferozepur and history of recent vaccination (HS) at other farm (Patiala). Outbreaks were reported in the months of May (Ferozepur) & June (Patiala). Tick infestation was also observed in animals. Ticks were collected from animals of both the farms, mounted in Canada balsam after clearing as per standard procedure and identified microscopically according to keys given by Miranpuri (1979). Post mortem of two dead animals (one at each farm) was also conducted on the spot. Gross lesions were recorded and heart blood was collected for microbiological culture examination.

PCR for A. marginale targeting msp1βgene: DNA from blood samples was extracted using Qiagen Blood Genomic DNA Extraction Kit as per the standard protocol of the manufacturer. The oligonucleotide primers targeting the *msp1*β gene of *A. marginale* included forward primer BAP-2: 5' GTA TGG CAC GTA GTC TTG GGA TCA 3 and reverse primer AL34S: 5' CAG CAGCAG CAA GAC CTT CA 3'(Ulrike *et al.,* 2003). The reaction mixture 25 µl constituted 12.5µl of KAPA 2G ^{TM} Fast Hotstart Ready Mix (2X containing KAPA2G fast hotstart DNA polymerase, KAPA 2G fast hotstart PCR buffer, 0.2 mM dNTP each, 1.5mM $MgCl_2$) with 1.5 µl/0.6 µM of BAP-2/ AL34S primers (10 pmol), 4.5µl nuclease free water and 5µl DNA template in automated Thermal cycler (Veriti 96 well thermal cycler, Applied Biosystems) with the following programme: initial denaturation at 95°C (5min), 30 cycles of denaturation at 95°C (1min), annealing at 60°C (1min), extension at 72° C (1.5 min) with final extension at 72 °C (5min). The amplified PCR products were separated by electrophoresis on 1% agarose gel and visualized under UV transilluminator for detection of 407 bp amplified product in positive cases. A sample with heavy parasitemia was used as positive control for standardization of PCR assays.

Statistical Analysis: Data was analyzed using SPSS software.

RESULTS AND DISCUSSION

Out of total 260 cattle at risk (218; Ferozepur and 42; Patiala), 40 (32; Ferozepur and 8; Patiala) were ill and 9 (6; Ferozepur and 3; Patiala) died of disease with morbidity, mortality and case fatality rate of 15.38%, 3.46% and 22.50%, respectively.

Out of 40 animals, 31 (77.5%), 4 (10%) and 5 (12.5%) were adult, heifers and calves, respectively. Major clinical symptoms observed in affected animals were pale mucus membranes, severe jaundice, increased respiratory rate and fever. Drop in milk production was also observed in lactating animals. Clinical symptoms observed in all animals except 2 adults and 5 calves, in which only fever was observed as the predominant sign of disease. Moderate to heavy tick infestation was observed in animals.

Examination of stained blood smears (n=15; 11 diseased and 4 healthy) was carried out for haemoparasites. Blood smears from healthy animals (n=4) were found negative for haemoparasites, where as 7 blood smears were found positive for *A. marginale* (Fig. 1*)*. Out of these 7

parasitologicaly positive animals, 6 were exhibiting severe jaundice and fever.

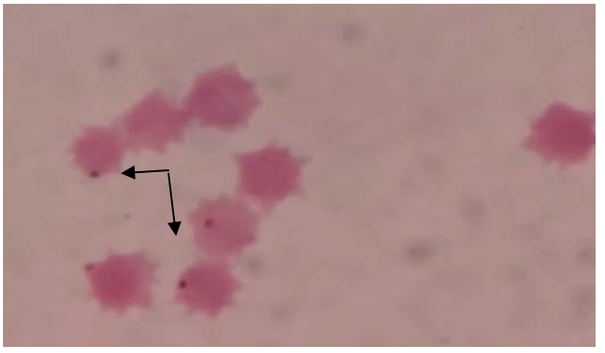

Fig. 1: Microphotograph of *A. marginale* in cattle blood: Leishman stained thin blood smear (x100)

PCR was employed on DNA extracted from all the 15 collected samples (11 diseased and 4 healthy animals). A total of 11 samples were found positive for *A. marginale* DNA as evident from agarose gel (1.5%) electrophoresis showing 407 bp fragment of amplified DNA/PCR product (Fig. 2). All the 11 positive samples were of diseased animals (including 7 microscopically positive). It indicates a higher sensitivity of PCR over traditional blood smear examination especially for detecting latent infections. Thin blood smears from infected animals with clinical sign of severe jaundice were already found positive for *A. marginale* by conventional microscopy, however positive results by molecular diagnosis (PCR) further confirmed the disease.

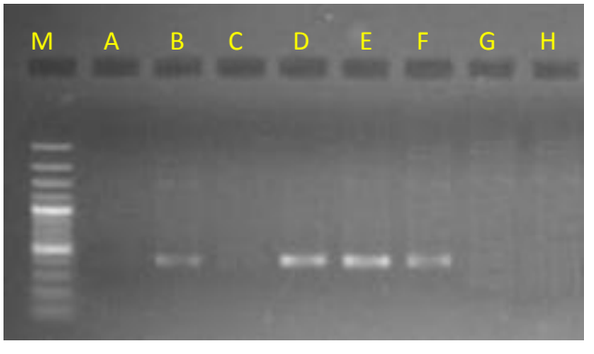

Fig. 2: Agarose gel (1.5%) electrophoresis showing 407 bp fragment of amplified *A. marginale* DNA from blood samples. Lane M: Molecular marker 100 bp, Lane A-D, F-G: Tested samples, Lane E: Positive control, Lane H: Negative control.

Postmortem of two animals (one at each farm) was also conducted on the spot. Major necropsy findings revealed splenomegaly, icterus and presence of thick granular bile in gall bladder. Jaswal *et al.* (2013) also observed similar postmortem lesions in cattle died of anaplasmosis. Heart blood collected from dead animals showed no microbial isolation on blood agar.

In this study, clinical findings of fever, jaundice, pale mucous membranes, anaemia, increased respiratory rate and decreased milk yield in infected animals pointed towards anaplasmosis. These signs were consistent with previous published reports of anaplasmosis in cattle resulted into mild to severe symptoms (Richey and Palmer, 1990; Birdane *et al*., 2006; Kocan *et al.,* 2010). Outstanding feature of clinical anaplasmosis is anemia associated with phagocytosis of parasitized erythrocytes. Jaundice is due to the destruction of the blood cells and release of their contents into the blood stream.

Cattle of all ages can become infected with *A. marginale*, however, in cattle over 2 years of age, the disease is acute and usually fatal, with risk of mortality 29–49% (Kocan *et al.,* 2010; Aubry and Geale 2011). Clinical signs are more severe in adult animals and infected calves usually do not show any clinical sign of the disease (Whittier *et al.,* 2009). Similar finding was observed in both the outbreaks, as among infected animals, majority were adults (77.5%), followed by heifers (12.5%) & calves (10%). Adult animals are considered to harbor carrier phase of the infection resulting from their prior exposures in life to *A. marginale* infection (Soulsby, 1982 and Singh *et al*., 2003). In the present study, all the infected animals with history of fever & jaundice were found positive for *A. marginale* infection both by conventional microscopy and PCR, however, animals with fever as only were detected positive by PCR. Usually pyrexia is the first recorded sign of anaplasmosis and may occur prior to the infection of 1% of the erythrocytes (Jones and Brock 1966).

In the present study, PCR based detection rate of *A. marginale* in infected cattle was found to be 100% as compared to 63.64% detection rate by conventional stained blood smear examination. PCR is found to be more sensitive as compared to conventional microscopy for diagnosis of infection in cattle, which has been previously reported by many authors (Carelli *et al*., 2007, Gale *et al.,* 1996 and Ashuma *et al.,* 2013, Jaswal *et al.,* 2014).

Table 1: Haematological parameters in parasitologicaly positive (Group I), Parasitologicaly negative and PCR positive (Group II) and healthy control (Group III) animals (Mean ±SD)

Group	Hb (g/dl)	TLC (per µl)	DLC (%)			
			N (%)	L(%)	M(%)	E(%)
I (n=7)	6.18 ± 2.17*	8571.43 ± 1819.51	48 ± 13.49	48.86 ± 12.78	2.71±2.43	0.43±0.73
II (n=4)	7.60 ± 0.51*	6375 ± 540.25	30 ± 13.93	69 ± 13.30	0.5±0.87	0.5 ± 0.87
III (n=4)	9.7 ± 0.83	8475 ± 944.39	38 ± 14.35	60 ± 13.34	0.5 ± 0.87	1 ± 1

*Statistically significant (P<0.05) as compare to control group (Group III).

Anaplasmosis is essentially a disease of adult cattle, while younger animals may remain susceptible but exhibit little detectable signs.

The hematological parameters showed significant (P<0.05) decrease in Hb (6.18± 2.17g/dl) in group I (microscopically positive, n=7) and group II (microscopically negative & PCR positive, n=4; 7.60±0.51g/dl) animals as compared to group III (healthy control, n=4; 9.7±0.83g/dl) (Table 1).

Affected animals were successfully treated with oxytertacycline, haematinics and antipyretics. However, due to advanced stage of disease, two animals died of disease, despite treatment. Ticks collected from both the outbreaks were identified as *Rhiphicephalus (Boophilus) microplus*. Singh and Rath (2013) also reported *R. (Boophilus) microplus* as predominant tick species in various agroclimatic zones of Punjab state. Studies conducted by Esteves *et al.* (2009) demonstrated that *R. (Boophilus) microplus* and *R. annulatus* may be the major tick vectors of *A. marginale* in tropical and subtropical regions of the world. Outbreaks were recorded in the month of May & June that are the conducive for multiplication of the tick vector (*R. (B.) microplus)* identified in the present study.

Although majority of haemoparasitic disease outbreaks occur during hot and humid weather (summer/rainy season) due to abundance of vector population, yet anaplasmosis outbreaks can occur at any time of the year. The various ways of transmission and the potential for carrier animals usually makes the source of an outbreak confusing. If an outbreak occurs during hot humid weather, it suggests that the source of the infection is from arthropod vectors. If an outbreak occurs at other times, newly purchased animals or increased stress should be considered as the source of the disease. When any outbreak occurs, it suggests that carrier animals are present either in herd, or neighboring herds, as carrier animals are an efficient source of infection (Whittier *et al*., 2009). There was history of purchase of new animals in Ferozepur district, however, proper quarantine measures were not followed at the farm. It could be the possibility that newly purchased animals may be in the carrier stage/incubation phase of disease. These animals may be responsible for outbreak at the farm. There was history of HS vaccination at other farm. It could be possibility that single needle could be used for vaccination in some animals, which resulted in spread of infection from carrier to healthy animals. Disease can also be transmitted mechanically when red blood cells infected with *A. marginale* are inoculated into susceptible cattle (Richey *and* Palmer 1990; Whittier *et al.,* 2009).

Livestock owners were advised to follow quarantine measures before mixing new animal into the existing herd. They were also advised for tactical acaricidal treatments for effective tick control. Moreover, they were also advised for rotation of drugs and to administer proper dosage to prevent problem of acaricidal resistance. Local veterinarians were also informed about the outbreaks for implementation of control measures to prevent the spread of disease among other animals in the surrounding areas.

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