

OCCURRENCE AND TRANSPLACENTAL TRANSMISSION OF *Anaplasma marginale* IN DAIRY CATTLE

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Abstract: Bovine anaplasmosis, caused by *Anaplasma marginale*, is a non-contagious tick borne disease. The main objective of the current study was to investigate comparative frequency of anaplasmosis in three different cattle breeds (European breed/Holstein Friesian and Jersey breed, indigenous breed and cross breed (1st and 2nd pedigree) and transplacental transmission using real time polymerase chain reaction for detection. Of the total 96 blood samples analyzed, our results indicated an overall incidence 45.83% (44/96) of *A. marginale* with highest incidence 62.5% (20/32) in European breed, followed by 42.4% (14/33) in cross breed and 35.4% (11/31) in indigenous breed. Most importantly, our results indicated that 13.7% (4/29) calves were found positive for the presence of *A. marginale* indicating transplacental transmission. Furthermore, indirect ELISA revealed an overall incidence rate of 34.3% (33/96) more likely indicating current or previous exposure. Finally, Giemsa staining determined that 15% (15/96) animals were found positive by examining red blood cells. Statistical analysis showed significantly higher ($P < 0.05$) incidence of European breed as compared to crossbreed and indigenous breed of cattle, while non-significant ($P > 0.05$) difference was found among the crossbred and indigenous breed of cattle. Moreover, non significant ($P > 0.05$) effect of age group was observed on the incidence of *A. marginale*. White blood cell count and mean corpuscular volume were significantly ($P < 0.05$) higher in infected cattle, while, red blood cells, packed cell volume hemoglobin concentration, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration (MCHC) were significantly ($P < 0.05$) higher in non-infected as compare to infected animals.

Key words: *Anaplasma marginale*; cattle breeds; enzyme linked immuno sorbent assay; real-time polymerase chain reaction; transplacental transmission

Introduction

Anaplasmosis is caused by *Anaplasma marginale*, an intra-erythrocytic rickettsial organism (1, 2). Bovine anaplasmosis alone, or in combination with babesiosis, is responsible for major economic losses to cattle farming due to adverse affects on the performance of cattle (3). Though exact estimate of economic losses due to anaplasmosis

in the study area have not been documented but an estimated annual loss due to anaplasmosis in the US alone amounts to \$100 million and includes 50000 to 100000 cattle deaths (4). In fact, anaplasmosis causes weight and production loss, delays growth, lowers fertility in bulls, abortions in females and may consequently lead to death (5, 6). It has been projected that tick borne diseases cause US \$ 13.9- 18.7 billion losses annually and about 80% population of cattle is at danger of tick borne diseases throughout the world (7).

Transplacental transmission of anaplasmosis is well documented in addition to its transmission by tick saliva after replication within the tick gut or mechanically through biting flies and contaminated fomites (8, 9). *Anaplasma marginale* may take 7-60 days for its incubation. Around 10- 90% of the red blood cells possibly will be parasitized in the severe condition of the diseases depending upon the *Anaplasma* strains and exposure of the host. Generally, all ages of cattle can be infected by *Anaplasma marginale*, but the intensity of the disease is age dependent. It has been previously observed that generally young calves were found less susceptible to clinical infection, but those between 1 and 2 years of age suffer from acute form and often become fatal with mortality range of 29 to 49% (10). Fluctuated intensity of red blood infected circulating cells has been observed between the 10-14-days of infection periods (11). *Anaplasma marginale* infected cattle will remain carrier and will transmit infection to susceptible cattle throughout their entire life (12).

Both direct and indirect methods are used for the diagnosis of anaplasmosis, whereas, direct microscopy is used for routine screening, however, DNA based detection through polymerase chain reaction (PCR) is highly sensitive.

Indirect enzyme-linked immunosorbent assay (ELISA) is a widely used technique for detecting and quantifying antibodies in serum samples, especially during epidemiological studies showing a sensitivity of approximately 96.9% (13). In Pakistan, *A. marginale* has been reported from various parts of Punjab (14, 15) and Sind province (16), but the issue has been under reported from parts of Khyber Pakhtunkhwa Pakistan. Furthermore, there has been increasing reports of susceptibility of European breeds to anaplasmosis infection. Moreover, current trends of ticks control have been mainly focused on to restrict transmission through vectors, while no attention is given to the parental screening for the carrier status. The current project was thus designed with an aim to evaluate the overall incidence of bovine anaplasmosis using sensitive detection tool such as real time PCR, to compare the susceptibility levels of European breed and indigenous breeds and to find the extent of transplacental transmission.

Materials and methods

Selection of animals

The current study was carried out during September 2014 and May 2015. Blood samples were collected from cattle breeds that were either indigenous (n=31), European breed (Jersey and Friesian; n=32) and cross-bred (indigenous cross European; n=33) of age groups 0-12 month, 13 months - 3years and over 3 years, respectively. Out of the total 96 animals, 67 were non pregnant and 29 were pregnant (last trimester). The pregnant animals were followed until parturition and calf was sampled at day 1.

Ethics and animals rights

The study was approved by the ethical committee of the university, The University of Agriculture, Peshawar Pakistan and all procedures were essentially carried out according to ethical rights laid down in 1964 Declaration of Helsinki and its later amendments.

Collection of blood samples and hematology

Fresh blood from both ear and caudal vein of each animal was collected. A total of 10 ml of blood from jugular vein of each animal was collected to isolate serum (in non EDTA containing vacutainer tubes); hematology plus PCR (in EDTA containing vacutainer tubes). Blood was collected from pregnant cows during last trimester and from new born calves on day 1st of birth. Collected samples were kept dry and were processed in the Laboratory of Parasitology at the department of Animal Health as well at Veterinary Research Institute (VRI), Peshawar for microscopic examination and PCR analysis. Serum was separated by centrifugation at 3000 rpm for 10 min after incubation at room temperature, and isolated serum was stored at 4-8°C. The EDTA containing blood was also stored at 4°C and shifted to VRI, Peshawar within two hours for hematology and real time PCR analysis. The hematology was performed through hematology analyzer URIT-2900 VET PLUS (China).

Preparation of blood Smear

Two thin blood smears were made from each blood sample and stained with Giemsa stain after drying for microscopic confirmation of *A. marginale* as described earlier (17) and was examined under the microscope. Samples were regarded positive based on finding of the dark blue dot shaped bodies (organism) in the margins of red blood cells using 100x oil emersion lens (18).

Serological detection of A. marginale

An indirect ELISA kit (SVANOVIR® *A. marginale* -Ab, Uppsala, Sweden) was used to detect antibodies against *A. marginale* in collected blood-serum according to the manufacturer's instruction.

Real Time PCR for Anaplasma marginale detection in cattle

Chromosomal DNA of *A. marginale* was isolated using DNA isolation kit DN easy® blood and tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Real time PCR was performed as described earlier (18) with slight modifications. In this PCR the major surface protein (msp1α) gene, a stable and ubiquitous gene marker specific to *A. marginale*, was targeted using primers combination: Forward primer TTGGCAAGGCAGCAGCTT and Reverse primer: TTCCGCGAGCATGTGCAT. The template DNA samples (1-5µg/µL) were mixed with 15 µL of Ssofast super mix and 2.5 µL of 10 pmol of each Primer. In addition, positive control (15 µL of Ssofast super mix (SsoFast TM®-Super Mix-BioRad, USA) + 5 µL of DNA + 2.5 µL of 10 pmol of each Primer) and negative control (15 µL of Sso fast super mix + 5 µL of PCR water + 2.5 µL of 10 pmol of each primer) were also added for the PCR reaction in CFX96 thermal cycler (BioRad, Hercules, CA, USA). The real time PCR conditions were set to (i) Initial denaturation at 95 °C for 1min (ii) cyclic denaturation at 95 °C for 1 min (iii) annealing at 60 °C for 1min (iv) extension at 72°C for 1 min and (v) final extension at 72 °C for 5 min. Primers were synthesized by Invitrogen.

Statistical Analysis

Data was nalyzed using SPSS version 16.0 to determine significant differences of *A. marginale*

occurrence among breed and age groups of both pregnant and non-pregnant cattle. Logistic regression was used specifically for knowing the frequency of *A. marginale* incidence. Means of various blood parameters were compared through ANOVA using General Linear Model and were ranked using Duncan's Multiple Range Test.

Results

Incidence of A. marginale in different cattle breeds

To determine the overall incidence of *A. marginale*, we performed real time PCR targeting gene msp1α as described earlier (18). Our results indicated that 44/96 (45.83%) samples were found positive for *A. marginale* indicating a high prevalence (Table 1). We further tested these samples with two other commonly used diagnostic techniques such as indirect ELISA and direct examination of blood smear with Giemsa stain to confirm exposure and sub/clinical form to observe the presence of parasite in the blood smear. Our results indicated that using ELISA, an overall 33/96 (34.3%) samples were identified positive, while only 15/96 (15.63%) could be declared positive using direct thin smear microscopy approach (19) (data not shown) indicating that these animals were sick of anaplasmosis. Secondly, this also indicates, that real time PCR is more sensitive than the traditional ELISA and blood smear techniques. Overall, our results indicated high prevalence (45.83%) of bovine anaplasmosis in cattle and high sensitivity, as expected, of real time polymerase chain reaction (RT-PCR) detection as compared to ELISA and direct microscopy.

Breed wise susceptibility

Our results indicated that, based on the RT-PCR, 20/32 (62.5%) European breeds, 14/33 (42.4%) of cross breeds and 11/31 (35.4%) indigenous breed were found infected with *A. marginale* (Table 1). Importantly, European breeds were living in a separate shed although within the same premises of the cross breeds. However, most of the indigenous breeds that were sampled were of small herd and routinely kept by the local farmers. Statistical analysis showed significantly

higher ($P < 0.05$) incidence of *A. marginale* in European breed as compare to crossbred and indigenous breed of cattle, while non-significant ($P > 0.05$) differences were found among the crossbred and indigenous breed of cattle (Table 1 and 2). Furthermore, statistical analysis showed no significant ($P > 0.05$) effect of breed age group on *A. marginale* incidence (Table 2).

Age wise susceptibility

Interestingly, our results indicated that age group 1 (0-12 months) of European breeds (Frisian and Jersey) were 70% infected, followed by age group 2 (13-36 months) and 3 (>36 months), respectively. However, in cross breed animals, 54.5% of the age group 2, 41.6% of age group 3 and 30% of age group 1 was infected. Finally, of the indigenous breeds, 40% of each group 2 and 3, while 27.2 % of age group 1 was infected (Table 1). Interestingly, statistical analysis indicated that no significant ($P > 0.05$) effect of age group was observed on the incidence of *A. marginale* in the age groups of various breeds of cattle (Table 2).

Above table indicates the incidence of *A. marginale* in cattle by using real-time PCR. Data indicate that exotic breeds were found highly susceptible, followed by cross and local breeds, respectively. Age group 1: 0-12 months, age group

2: 13-36 months, age group 3: over 36 months.

Above table indicates different blood parameters of infected vs non-infected cattle breeds. Mean of the total of 44 positively diagnosed cattle vs all mean of the 52 negatively diagnosed cattle. Different superscripts within the row are significantly different at p-value (0.05).

Effects on Hematological parameters of infected cattle

Hematological parameters indicated significant differences ($P < 0.05$) among the infected and non-infected animals. White blood cell count (WBC) and mean corpuscular volume (MCV) were significantly ($P < 0.05$) higher in infected as compared to non-infected animals. While red blood cells (RBC), packed cell volume (PCV), hemoglobin concentration (HGB), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were significantly ($P < 0.05$) higher in non-infected as compare to infected animals (Table 3).

Transplacental transmission of *A. marginale* from infected cattle

We examined a total of 29 day-old calf of already positively declared (for *A. marginale*)

Table 1: Incidence of *A. marginale* in different cattle breeds

Breed	Age group	No. of samples examined	No. of Positive samples	Incidence of <i>A. marginale</i> (%)
HF/Jr	1	10	7	70
	2	10	5	50
	3	12	8	66.66
	Total	32	20	62.5
Cross	1	10	3	30
	2	11	6	54.54
	3	12	5	41.66
	Total	33	14	42.4
Local	1	11	3	27.27
	2	10	4	40
	3	10	4	40
	Total	31	11	35.4

Above table indicates the incidence of *A. marginale* in cattle by using real-time PCR. Data indicate that exotic breeds were found highly susceptible, followed by cross and local breeds, respectively. Age group 1: 0-12 months, age group 2: 13-36 months, age group 3: over 36 months.

Table 2: Frequency distribution of *A. marginale* in different cattle breeds

Age/Breed	Score	D F	P. Value
Breed(1)	4.706	1	0.030
Breed(2)	0.400	1	0.527
Breed(3)	5.015	2	0.081
Age group(1)	0.466	2	0.792
Age group(2)	0.449	1	0.503
Age group(3)	0.042	1	0.838
Age group * Breed	3.822	4	0.431
Age group(1) by Breed(1)	2.397	1	0.122
Age group(1) by Breed(2)	1.276	1	0.259
Age group(2) by Breed(1)	0.044	1	0.834
Age group(2) by Breed(2)	0.294	1	0.588

Above frequency distribution results are based on the outcome of real time PCR as depicted in Table 1. Breed 1 = Pure exotic; 2 = Crossbred; 3 = Local breed; Age group 1: 0-12 months; Age group 2: 13-36 months; Age group 3: over 36 months.

Table: 3 Hematological parameters of infected and non-infected dairy cows by *A. marginale*

Parameters	Non infected Mean \pm SE	Infected Mean \pm SE	P- Value
WBC (10 ⁹ /L)	10.79 ^b \pm 0.06	11.81 ^a \pm 0.13	0.001
RBC (10 ¹² /L)	8.08 ^a \pm 0.13	4.96 ^b \pm 0.35	0.001
HGB (g/dL)	9.93 ^a \pm 0.10	7.76 ^b \pm 0.28	0.001
PCV (%)	32.72 ^a \pm 0.19	27.12 ^b \pm 0.10	0.001
MCV (f/L)	45.29 ^b \pm 0.05	47.86 ^a \pm 0.02	0.002
MCH (p/g)	15.89 ^a \pm 0.01	14.24 ^b \pm 0.05	0.001
MCHC (g/dL)	35.30 ^a \pm 0.06	33.07 ^b \pm 0.10	0.001

pregnant cattle by RT-PCR. Our results indicated that 4/29 (13.7%) of calves were found infected with *A. marginale* indicating reasonable level of transplacental transmission.

Discussion

Bovine anaplasmosis caused by *A. marginale* is a major constraint on livestock production and health of cattle due to its devastating economic impact on the production potential of cattle. Incidence cases of *A. marginale* in diverse range of cattle breeds and evidences of transplacental dissemination from Khyber Pakhtunkhwa, province of Pakistan have been rarely reported. In Pakistan including Khyber Pakhtunkhwa, import of European breeds such as Jersey and Holstein Friesian have been enormously

increased during the last five years. Although, the exact data of the number of European breeds in Khyber Pakhtunkhwa does not exist, but based on our own personnel perception and raw data collected from organized commercial farms and breed centers indicated a significant number of European breeds in the province. Although, it is not of surprise that these European breeds would be comparatively more prone to infectious diseases and climatic alterations, however, exact data of such information is lacking in the country and particularly in Khyber Pakhtunkhwa although using all preventive measurement like anti-tick spray. The current study thus certainly gave a first brief overview of the comparative susceptibility of European and indigenous /cross breeds towards *A. marginale* and necessitates the need to ensure and provide safe, clean and supportive environment

in order to draw benefit from European breeds at maximum of their potential. In the current report, we screened a total of 96 blood samples for the presence of *A. marginale* in different cattle breeds in order to determine its incidence, comparative susceptibility of breeds and age of cattle breeds and transplacental transmission. We report on the high occurrence of *A. marginale* particularly in the European breeds and further report on the evidence of transplacental transmission.

Detection of *A. marginale* by PCR is considered highly sensitive and accurate that could even detect the carrier stage in contrary to the classical methods such as direct microscopy and ELISA. Although, there are less expensive alternatives, such as serological tests (ELISA, IFAT) and direct microscopy, but these methods lack specificity and sensitivity as compared to PCR for the detection of *A. marginale*. Moreover, these methods are not able to reveal the exact profile of prevalence of infection at a particular point, as detectable levels of antibodies may remain in the animal for long periods, even after elimination of the infectious agent. As indicated by our current studies a low level 35.41% of detection of *A. marginale* by ELISA and 15.62% detection limit by direct microscopy was observed, corroborated by previous findings (20, 21). Molecular techniques such as PCR, on the other hands due to its higher sensitivity and specificity, indicated higher prevalence of 45% in the current study and 56.9% in Puntarenas Province, Costa Rica (22). Similarly, a prevalence of 48.75% was observed earlier (23).

A. marginale was found significantly ($P < 0.05$) higher in the European breeds in comparison to crossbreeds and indigenous breeds of cattle. While non-significant ($p > 0.05$) differences were found among the indigenous and crossbreeds of cattle. Similar study was conducted by Marufu et al. (24) who revealed significantly higher incidence of anaplasmosis in European breed (pure Frisians) as compared to the cross and indigenous breeds of cattle. Our findings are also supported by Alim et al. (25), who revealed lower prevalence of anaplasmosis in indigenous cattle as compare to Holstein Frisian cattle, however, there was no significant difference of *A. marginale* prevalence among different age groups of cattle as described earlier. Our findings are also supported by Hamou et al. (26) that described no significance difference of *A. marginale* among difference age group of cattle. It is known that breed improvement for high

production of milk and meat may compromise the immune system of the genetically improved animals (27) most likely making them vulnerable to infectious diseases.

WBC and MCV were significantly ($P < 0.05$) higher in infected as compared to non-infected animals. However, RBC, HGB, PCV, MCH and MCHC were significantly ($P < 0.05$) lower in infected as compared to non-infected cattles. The lower number of RBCs observed is more likely due to the activated immune system against the parasitized cells to eliminate them from the body. Since, destruction of RBCs occur more rapidly due to phagocytosis of the infected RBCs and demand of the body increase for RBC resulting selective pressure to release immature RBCs from bone marrow. Because, the immature RBCs harbor larger size than mature red blood cells so resulting in increased MCV (28).

Transplacental transmission of *A. marginale* has been reported previously with a wide range indicating varying degree of vertical transmission in different animals (29, 30). We indicated a 4% transplacental transmission following those animals that were detected anaplasma positive during their pregnancy. However, in most of the cases, we were not sure of the exact day and time of infection though. Other reports indicated a comparatively higher incidence (12.5 %) in cattle calves, but they did not mention the status of the diseases in the mother and the time of the infection when the mother was infected. The feature of uterine transfer has implication in the epidemiology of anaplasmosis in infection free areas. Interestingly, we observed a comparatively lower prevalence of transplacental transmission of *A. marginale* in infected pregnant cattle to their calves as compared to previous reports of 10.5%-12.5% (31,32) Similarly, in South Africa, a transplacental transmission was found to be 15.6% among calves borne from cows showing chronic infection or primo-infected during pregnancy (33), however, from clinically infected animals while showing clinical signs, transplacental transmission was noted considerably as high as 86.4% (32/37) in calves (34). The mechanism of transplacental transmission needs further investigation to understand its dissemination. The higher transplacental transmission rate may be due to the clinical or acute infection of anaplasmosis.

European breeds (Holstein Friesian and Jersey) were found more susceptible to *A. marginale* as

compared to the cross (1st and 2nd pedigree) and indigenous breeds. Using RT-PCR indicated a higher 45.83% (44/96) prevalence of *A. marginale* with highest incidence of 62.5% (20/32) in European breeds, followed by 42.4% (14/33) in cross breeds and 35.4% (11/31) in indigenous breeds. ELISA indicated that 35.4% had exposure to the parasite, while 13.7% were found positive for parasite in their blood samples. Finally, detection of *A. marginale* in day-old calves (pure exotic breeds) indicated its ability to transmit vertically.

Acknowledgment

There was no financial sponsor to the study. However, we acknowledge the staff members and management of Veterinary Research Institute, Peshawar, Pakistan for providing equipment facility to perform RT-PCT.

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POJAVNOST IN PRENOS *Anaplasma marginale* PREKO POSTELJICE PRI MLEČNEM GOVEDU

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Povzetek: Goveja anaplazmoza, ki jo povzroča *Anaplasma marginale*, je bolezen, ki ni kužna in jo prenašajo klopi. Glavni namen študije je bil raziskati in primerjati pogostost anaplazmoze pri različnih pasmah goveda in sicer dveh evropskih pasmah (holstein-frizijske pasma in pasma jersey) pri avtohtoni pasmi ter pri križancih med evropskimi pasmami in avtohtonimi pasmami (1. In 2. generacija križancev) ter prenos preko posteljice z uporabo verižne reakcije s polimerazo v realnem času (PCR). Od skupno 96 pregledanih vzorcev krvi so rezultati pokazali skupno incidenco 45,83 % (44/96) *A. marginale* z največjo incidenco 62,5 % (20/32) pri evropskih pasmah, nato 42,4 % (14/33) pri križanih živalih in 35,4 % (11/31) pri avtohtonih pasmah. Rezultati so tudi pokazali pozitivno reakcijo na prisotnost *A. marginale* pri 13,7 % (4/29) telet, kar kaže na prenos anaplazmoze preko posteljice. Metoda posrednega testa ELISA, ki pokaže na trenutno ali predhodno izpostavljenost, je pokazala 34,3-odstotno pojavnost pri vseh živalih skupaj (33/96 živali). Proučitev rdečih krvničk z barvanjem Giemsa je pokazalo 15 % (15/96) pozitivnih živali. Statistična analiza je pokazala statistično značilno razliko v pojavnosti anaplazmoze med evropskima pasmama v primerjavi s križanci in avtohtono pasmo ($p < 0,05$), razlika med križanci in avtohtono pasmo goveda pa ni bila statistično značilna. Poleg tega je bil pri pojavnosti *A. marginale* opazen neznaten (statistično neznačilen) učinek starosti proučevanih živali. Število belih krvnih celic in povprečni volumen telesne mase sta bila pri okuženih govedih statistično značilno povečana ($p < 0,05$), medtem ko so bile rdeče krvne celice, koncentracija hemoglobina v celicah in povprečna količina hemoglobina v posameznem eritrocitu (MCHC) v neokuženi skupini višja kot v skupini z okuženimi živalmi ($p < 0,05$).

Ključne besede: *Anaplasma marginale*; pasme goveda; test ELISA; verižna reakcija s polimerazo v realnem času; prenos preko posteljice