Research article

Prevalence of anaplasmosis in cattle from Chattogram Division of Bangladesh

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A R T I C L E   I N F O

A B S T R A C T

Bovine anaplasmosis is an important haemoparasitic disease of farm animals associated with significant economic loss. The current study was designed to determine the prevalence and associated risk factors and molecular characterization of *Anaplasma* species. A total of 1680 blood (crossbred=455, local=1225) from cattle of selected hilly, coastal and plain areas of Chattogram Division. Bangladesh. The conventional microscopic examination using the Giemsa staining technique was considered to examine the samples. A total of 50 randomly selected blood samples (apparently positive as determined by microscopy) were tested using polymerase chain reaction (PCR) to confirm the disease. Partial genome sequencing (based on *16S rRNA* gene) and phylogenetic analyses were performed. Results demonstrated that a total of 8.21% of cattle (n=1680) were positive for *Anaplama marginale* infection. The prevalence of anaplasmosis in the hilly area was 9.33% followed by coastal area (9.00%) and plain area (5.83%). The prevalence of *Anaplasma* spp. was higher (P>0.05) in crossbred compared to indigenous cattle. The infection was significantly higher (P<0.05) in winter (11.09%) followed by rainy (7.13%) and summer (6.43%) season. Animals aged between 18-30 months were found to be more infected to anaplasmosis. The prevalence of anaplasma infection was higher in female animals compared to males. Amplification of *16S rRNA* gene showed 80% (40 out of 50 samples) of the samples were found positive for *Anaplama marginale* infections. Phylogenetic studies revealed that the isolate from this present study was closely related to the isolates of Argentina, Australia and China.

1. INTRODUCTION

Anaplasmosis is an economically important vector borne hemoparasitic disease of cattle aused by the rickettsial organism of the genus Anaplasma (Order Rickettsiales, Family Anaplasmataceae). It commonly affects cattle, sheep, goat, buffalo and some wild ruminants and occurs in tropical and subtropical regions worldwide. In bovine species, it is transmitted mechanically and biologically (ticks). Cattle of all ages are susceptible to anaplasmosis but

clinically significant in young cattle. Anaplasmosis is characterized by fever, weight loss, decreased milk production, pale mucus membranes, severe anaemia, jaundice, brownish urine, hyper- excitability, abortion. Sometimes, the mortality is seen without showing any sign of hemoglobinemia and hemoglobinuria during acute form of the disease. It causes economic losses in the livestock industry in the form of death, weight loss, chronic case, abortion, production loss and associated veterinary expenses.

Epidemiological data largely relies on efficient diagnosis of blood parasites which is crucial for their effective prevention and control. Different diagnostic methods are used to diagnose anaplasmosis, such as classical Giemsa-stained blood smear examination, serological tests and molecular tests (Aubry and Geale, 2011). Different molecular methods targeting different genes were used for the detection of *Anaplasma* spp. The most commonly used molecular markers for detection of *A. marginale* are 16S rRNA (Zhou et al., 2010), heat-shock protein (groEL) (Lew et al., 2003) and major surface protein (MSP) (Molad et al., 2004). *MSP4* genes have been used for phylogenetic studies providing information on biogeography and evolution of *Anaplasma* spp (Torina et al., 2012). Different climatic conditions at different geographical areas of Bangladesh greatly affect the multiplication of vectors that contribute to the disease spread. Until now, only a few parasitological and molecular investigations covering plain, hilly and coastal areas were carried out on commonly available blood parasites in different regions of Bangladesh (Alim et al., 2012; Siddiki et al., 2010; Samad et al., 1989).

During this study, we used routine parasitological examination of peripheral blood and molecular techniques (based on PCR amplification of 16s ribosomal subunit gene and *MSP4* gene) to accurately identify positive cases of anaplasmosis in study areas with a view to further molecular characterization and phylogenetic analyses. The present study was undertaken to determine the prevalence and risk factors of anaplasmosis in the hilly, coastal and plain areas of Chittagong Division. The molecular identification and phylogenetic origin of identified *Anaplasma* spp. were also performed using PCR followed by sequencing phylogenetic analysis.

2. MATERIALS AND METHODS

Study area and seasons

The study was conducted in the hilly and the coastal areas of 4 different southern districts of Bangladesh. These include Chattogram Metropolitan area (plain areas), Bandarban sadar and Ruma upazila of Bandarban district, Rangamati sadar and Kawekhali upazila of Rangamati district (hilly areas); Noakhali sadar and Suburnochar upazila of Noakhali district and Lakshmipur sadar and Ramgoti upazila of Lakshmipur district (coastal areas). The study was conducted for a period of 12 months.

Target animals

Holstein Friesian (HF) crossbred and local cattle (Red Chittagong /Indigenous/Non-descript) were selected as target animals for this study. To determine the age and breed susceptibility, cattle were classified into three subgroups. For HF crossbred cattle, it was calf (≤1 year), Young (>1 – < 2.5 year) and Adult (≥2.5years) and for local cattle, it was Young (>1-3.5 years) and adult cattle (≥3.5 years).

Demographic data and sample collection

In this cross-sectional study, a standard questionnaire was used to collect demographic data such as breed, age, sex, animal identification and floor type (e.g. cemented or mud) The period was divided into three seasons namely, i) winter (November to February), ii) summer (March to June) and iii) rainy (July to October).

Blood samples were collected at one point of every month from each selected area. An individual animal was considered as a sampling unit. A total of 1680 whole blood samples (600, 600 and 480 from hilly, coastal, and plain area, respectively) were collected. About 3-5 mL of blood was collected from the jugular vein in blood collection tube containing EDTA. Samples were preserved at -25^0 freezer until further use.

Microscopic examination

Two thin blood smears were prepared for microscopic examination and subsequently air-dried and fixed by 100% methyl alcohol for 3-5 min. The prepared thin blood smears were then stained with Giemsa stain for 25-30 min following previously described method (Urquhart et al. 1996).

DNA extraction and PCR assay

Total genomic deoxyribonucleic acid (gDNA) was extracted from the whole blood samples (N=50) by using the PCI method (Barbaro et al., 2004). PCR amplification was carried out using a 2720 thermal cycler (Applied Biosystems, USA). To identify the *Anaplasma* spp, PCR amplification of two genes, *16sRNA* and *MSP4* was performed using previously known primer pairs (Table 1).

DNA sequencing

PCR amplicons were purified using a commercial PCR purification Kit (Favorgen, Taiwan). The purified DNA was then measured by fluorimeter for concentration (ug/ul) before sending for DNA sequencing through commercial sources. Purified PCR products were used for sequencing by a commercial supplier (Bioneer Corp, South Korea) for DNA sequencing.

Phylogenetic analysis

The sequences were initially checked for sequence similarity using a BLAST from the NCBI (the National Center for Biotechnology Information: http://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple alignment analysis was performed using the clustalw program, while the phylogenetic analysis was performed by the (Saitou and Nei, 1987). The evolutionary distances were neighbor-joining method computed using the p-distance method using the MEGA software, version-5. The tree stability was estimated by a bootstrap analysis for 1,000 replications.

Statistical analysis

The obtained information was imported, stored and coded accordingly using Microsoft Excel-2003 to STATA/IC-11.0 (Stata Corporation College Station, TX, USA) for analysis. Significance was determined when $P<0.05$.

3. RESULTS

Prevalence of anaplasmosis in cattle

Conventional blood smear examination demonstrated that 138 were found positive (out of 1680 cattle) for *A. marginale* infection with a prevalence rate of 8.21%. Further observation based on the origin of samples indicated considerable variations among the prevalence of anaplasmosis in different geographic areas (Figure 1). The prevalence of anaplasmosis in the hilly area was 9.33% (56 out of 600), while in the coastal area, it was 9.00% and in the plain area 5.83%.
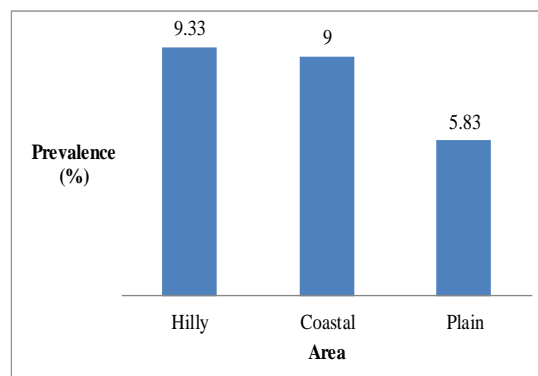

Figure 1. Prevalence of anaplasmosis in cattle in Chattogram division.

Table 1. list of primers used for amplification of DNA of *Anaplasma* sp.

Target gene	Primer	Sequence	Amplicon size (bp)	Reference
16SrRNA	AE-F	5'-AAGCTTAACACATGCAAGTCGAA-3'	1406	Oh et al., 2009
	AE-R	5'-AGTCACTGA CCCAACCTTAAATG-3'		
MSP4	AM-F	5'-TTGTTTACAGGGGGCCTGTC-3'	831	Molad et al., 2004
	AM-R	5'-GAACAGGAATCTTGCTCCAAG-3'		

Variable-wise prevalence is presented in Table 2. The prevalence of *Anaplasma* spp. was higher (P>0.05) in crossbred cattle compared to local cattle. The disease was significantly higher (P<0.05) in winter than rainy and summer seasons. Most of the *Anaplasma* infection occurred in animals aged 18-30 month (Figure 2). Animals kept on mud type floor seem to be more susceptible to ana-plasmosis than the animals kept on the cemented floor. Female animals had higher prevalence compared to their male counterparts.

Table 2. Variable-wise prevalence of ana-plasmosis in crossbred and local cattle in Chattogram division

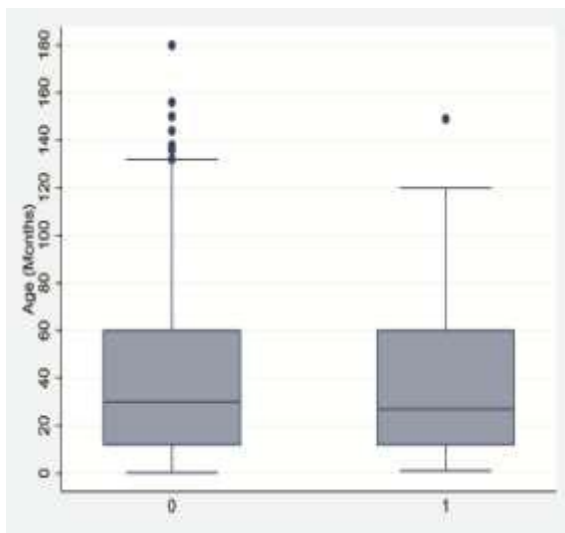| Variables	Categories (N)	Prevalence
Season	Summer (560)	6.43 (36)
	Rainy (561)	7.13 (40)
	Winter (559)	11.09 (62)***
Sex	Male(400)	6.50 (26)
	Female (1278)	8.75(112)
Floor	Cemented (1011)	7.42 (75)
	Mud-type (669)	9.42 (63)
Breed	Cross (455)	9.23 (42)
	Local (1225)	7.84 (96)

*** Significance at P<0.01

Figure 2. Box plot showing association between anaplasmosis with age, where zero (0) indicate negative and 1 indicate positive. The box plot indicates that 50% of infected animals were within the age group of 18-30 months.

Molecular detection of Anaplasma

The 50 PCR amplified samples tested, 40 out of samples were found positive for the 16S rRNA gene All positive samples for the 16S rRNA gene gave positive results in MSP4 gene screening (Figure 3 and 4). The PCR product (amplicon) was sent for sequencing and one sequence was curated and submitted to the NCBI database (GenBank Accession no. KX110079)
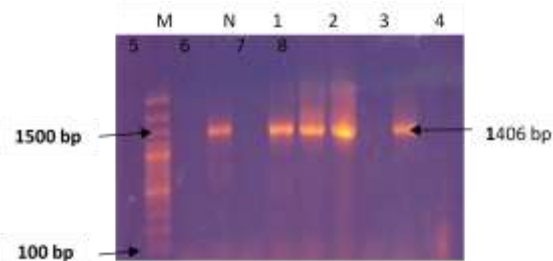

Figure 3. Result of PCR amplification for the *16SrRNA* gene of *Anaplasma marginale*. Lane M is for 100 bp plus DNA ladder; N is for negative control; Lanes 1-8 is suspected samples; Lanes 1, 3, 4, 5, and 7 having amplicons of ~ 1406 bp indicated presence of *A. marginale* organism.

Figure 4. Result of PCR amplification for the *MSP4* gene of *Anaplasma marginale*. Lane M is for 100 bp DNA ladder; N is for negative control; Lanes 1-14 is suspected samples; Lanes 1, 2, 3, 5, 7-14 having amplicons of 831 bp indicated presence of *A. marginale* organism.

Sequence similarity and phylogeny

The *MSP4* nucleotide sequences from the present study (Accession no. KX110079) was compared with other sequences published in GenBank and revealed 99% identities to *A. marginale* isolates from Australia, Mexico, Argentina, Venezuela, China, Brazil, Taiwan, Israel, Italy, Tunisia, Nigeria, Zimbabwe, North America and Spain. The phylogenetic tree for *MSP4* gene sequences of *A. marginale* isolates is shown in Figure 5. *A. marginale* CVASU

isolate formed a separate branch but clustered together with *A. marginale* strains originating from Argentina, Australia, Mexico, Venezuela and China.

Figure 5. Phylogenetic tree of the nucleotide sequences of *Anaplasma marginale MSP4* gene The accession numbers and countries are shown before isolate name. The fragment of *MSP4* gene sequences obtained in this study were indicated with bold triangle. The tree was inferred using the neighbor joining method of MEGA 5.2, bootstrap values are shown at each branch point. Number next to the branch demonstrates bootstrap support from 1000 replications. All sites of the alignment containing insertions-deletions, missing data were eliminated from the analysis (option "complete deletion").

4. DISCUSSION

The overall clinical prevalence of anaplasmosis in this study was 8.21%. With this finding differs previous studies that used samples from other geographic areas of Bangladesh (Chowdhury et al., 2006; Alim et al., 2012). A similar observation (9.71% prevalence) was reported by other researchers in neighboring Pakistan (Atif et al., 2012). The variations of the results could be due to random selection of apparently healthy animals rather than the clinically suspected animals. The highest prevalence of anaplasmosis was recorded in hilly areas during this study, followed by coastal areas and plain areas. The prevalence of anaplasmosis in the hilly areas was not consistent with a previously published report that recorded 14.94% of prevalence of anaplasmosis in the three hilly districts of Chittagong (Mohanta et al., 2013). It might be

due to the variation of the study areas. Higher prevalence in the hilly areas may be due to high medicine of vectors, poor management system of rearing and unavailability or negligence of using a drug to control vector populations.

The breed of animal is thought to have some impact on the incidence of bovine anaplasmosis. High incidence of anaplasmosis in crossbred cattle was recorded during this study compared to the indigenous or local cattle. However, this was not statistically significant. Nevertheless, the finding was consistent with the observation by other investigators who used animal samples from different parts of Bangladesh (Alim et al., 2012; Atif et al., 2012; Chowdhury et al., 2006; Rahman et al., 2015). This might be due to the lack of immunity during the high milk yielding stage and characteristic genetic makeup and seasonal stress in the summer months.

The present study could not find any statistically significant differences among male and female animals infected with *Anaplasma* spp. Yet, the percent prevalence was found to be higher in female animals compared to male animals. Higher prevalence in female may due to keeping them for breeding and milk production purpose for a more extended period, supplied insufficiently balanced ration against their high demand (Kamani et al., 2010). This finding was consistent the observation by other investigators (Alim et al., 2012; Atif et al., 2012). Analyses also indicates that animals aged 18-30 months are more susceptible to anaplasmosis. This observation is consistent with a previous report (Rahman et al., 2015) which recorded a high prevalence in animals aged between 12 and 30 months. However, one report indicated 2-3 years older animals to be more vulnerable to anaplasmosis (Atif et al., 2012) while, two other groups indicated adult animals are more susceptible than younger animals (Alim et al., 2012; Chowdhury et al., 2006). This may be due to weak immune system in young animals compared to adult and might be an immune consequence of clinical infection. Seasonal variation in disease incidence is considered one of the most influencing factors on the prevalence of blood parasitic diseases. This is mainly due to the environmental factors that regulate the tick population in the farm or adjacent areas. No meteorological data

(temperature, rainfall, humidity, etc.) were collected during the present study. However, we attempted to identify the variations in prevalence of the protozoan diseases among different seasons. The prevalence of anaplasmosis was found to be significantly higher in the winter season followed by the rainy and summer season. Similar seasonal variation was observed by previous investigators who reported a higher incidence in the winter season in cattle in the northern Sylhet district of Bangladesh (Nath and Bhuyian, 2013). However, contrary to this observation, many other studies have reported higher prevalence in the rainy and summer season (Alim et al., 2012; Belal et al., 2015). All these variations are thought to be due to macroclimate changes essential for breeding ticks (Vairamuthu et al., 2012).

The housing and floor type in animal houses was considered an essential factor in the incidence of anaplasmosis in animals. High incidence was recorded in conventional type farmhouses compared to animals that were kept in cemented (paka) floor with better management options. A similar findings was recorded by other researchers who suggested that the variation is due to higher vector load in muddy floor (Nath and Bhuyian, 2013; Sajid et al., 2014).

Although selected samples screened through microscopy were further analysed for molecular study, 80% of the samples were found positive in PCR assay based on the amplification of partial *16SrRNA gene*. The remaining negative samples might include false-positive cases where parasitic stages are frequently confused with other microscopic structures (inside RBCs) such as Heinz bodies, Howell-Jolly bodies or staining artefacts. Notable that, further PCR assay of *MSP4 gene* fragments also successfully validated the PCR results as both the genes were amplified in the same sample sets. Further validation of the genus was confirmed by DNA sequencing followed by sequence-similarity based BLASTn search. The *MSP4* gene-specific primer pair routinely used for identification of *A. marginale* was used during this study. The *MSP4* gene is highly conserved among different strains of *A. marginale* and also between different *Anaplasma* spp. Phylogenetic analyses of the *MSP4* gene fragment sequences demonstrated that the Bangladeshi *A. marginale* isolates share a separate branch but clustered with those from other countries. They were also positioned closer to China, Argentina, Australia, and Mexico as supported by low bootstrap value, indicating substantial genetic variations among them.

5. CONCLUSION

The present study attempted to determine the prevalence of anaplasmosis in cattle and molecular characterization of *Anaplasma spp*. The highest prevalence of anaplasmosis in the selected study areas indicated that it is one of the important parasitic diseases encountered by the farmers in selected areas of Bangladesh. Further studies focusing identification of tick vectors along with molecular detection of organisms from both vectors and hosts would be interesting to understand the actual genetic diversity of *Anaplasma spp*.

REFERENCES

Alim, M. A., Das, S., Roy, K., Masuduzzaman, M., Sikder, S., Hassan, M. M., Siddiki, A. Z., Hossain, M. A., 2012. Prevalence of hemoprotozoan diseases in cattle population of Chittagong division, Bangladesh. Pakistan Veterinary Journal, 32: 221-224.

Atif, F. A., Khan, M. S., Iqbal, H. J., Arshad, G. M., Ashraf, E., Ullah, S., 2012. Prevalence of *Anaplasma marginale*, *Babesia bigemina* and *Theileria annulata* infections among cattle in Sargodha district, Pakistan. African Journal of Agricultural Research, 7: 3302-3307.

Aubry P and Geale DW., 2011. A review of bovine anaplasmosis. Transboundary Emerging Diseases, 58(1): 1–30.

Belal, S. M. S. H., Al Mahmud, M. A., Ferdous, M. J., 2015. Prevalence of Anaplasmosis in cattle in Sirajganj district of Bangladesh. Research in Agriculture Livestock and Fisheries, 1: 97-103.

Chowdhury, S., Hossain, M. A., Barua, S. R., Islam, S., 2006. Occurrence of common blood parasites of cattle in Sirajgonj sadar area of Bangladesh. Bangladesh Journal of Veterinary Medicine, 4: 143-145.

Lew, A. E., Gale, K. R., Minchin, C. M., Shkap, V., de Waal, D. T., 2003. Phylogenetic analysis of the erythrocytic *Anaplasma* species based on 16S rDNA and GroEL (HSP60) sequences of *A. marginale*, *A. centrale*, and *A. ovis* and the specific detection of *A. centrale* vaccine strain. Veterinary Microbiology, 92: 145-160.

Kamani, J., Sannusi, a., Eqwu, O.K., Dogo, G. I., Tanko, S Kemza, S., AE Takarki, A. E., DS Gbise, D.S., 2010. Prevalence and significance of haemoparasitic infections of cattle in North-Central, Nigeria. Vet World., 3: 445-448

Mohanta, U., Atiquzzaman., Mondal, M. M. H., 2013. Tick and Tick Borne Protozoan Diseases of Livestock in the Selected Hilly Areas of Bangladesh. International Journal of Agricultural Research, Innovation and Technology, 1: 60-63.

Molad, T., Brayton, K. A., Palmer, G. H., Michaeli, S., Shkap, V., 2004. Molecular conservation of *MSP4* and MSP5 in *Anaplasma marginale* and *A. centrale* vaccine strain. Veterinary Microbiology, 100: 55-64

Nath, T. C., Bhuiyan, J. U., 2013. A Study on the Prevalence of Common Blood Parasites of Cattle in Sylhet District of Bangladesh. International Journal of Animal and Fisheries Science, 1: 8- 13.

Oh, J. Y., Moon, B. C., Bae, B. K., Shin, E., Ko, Y. H., Kim, Y. J., Park, Y. H., Chae, J. S., 2009. Genetic identification and phylogenetic analysis of *Anaplasma* and *Ehrlichia* species in *Haemaphysalis longicornis* collected from Jeju Island, Korea. Journal of Bacteriology and Virology, 39: 257-267.

Rahman, A. S. M. S., Sumon, S. M. M. R. , Khan, M. A. H. N. A., Islam, M. T., 2015. Current status of subclinical form of babesiosis and anaplasmosis in cattle at Rangpur district in Bangladesh. Progressive Agriculture, 26: 51-59.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425.

Sajid, M. S., Siddique, R. M., Khan, S. A., Iqbal, Z., Khan, M. N., 2014. Prevalence and Risk Factors of Anaplasmosis in Cattle and Buffalo Populations of District Khanewal, Punjab, Pakistan. Global Veterinaria, 12: 146-153

Samad, M. A., Bashar, S. A., Shahidullah, M., Ahmed, M. U., 1989. Prevalence of haemoprotozoan parasites in cattle of Bangladesh. Indian Veterinary Medical Journal, 13: 50-51.

Siddiki, A. Z., Uddin, M. B., Hasan, M. B., Hossain, M. F., Rahman, M. M., Das, B. C., Sarker, M. S., Hossain, M. A., 2010. Coproscopic and haematological approaches to determine the prevalence of helminthiasis and protozoan diseases of Red Chittagong Cattle (RCC) breed in Bangladesh. Pakistan Veterinary Journal, 30: 1-6.

Torina, A., Agnone, A., Blanda, V., Alongia, A., Agostino, R.D., Caracappa. S., 2012. Development and validation of two PCR tests for the detection of and Anaplasma marginale. Ticks and Tick-Borne Diseases, 3: 282-6

Urquhart, G., Armour, J., Duncan, J., Dunn, A., Jennings, F., 1996. Veterinary Parasitology2nd ed. Black well Science Ltd, Oxford, UK, pp. 213.

Vairamuthu, S., Pazhanivel, N., Suresh, R. V., Balachandran, C., 2012. *Babesia bigemina* infection in a 20 day old non-descript calf-a case report. Indian Journal of Field Veterinarians, 7: 69-70.

Zhou, Z., Nie, K., Tang, C., Wang, Z., Zhou, R., Hu, S., Zhang, Z., 2010. Phylogenetic analysis of the genus *Anaplasma* in Southwestern China based on 16S rRNA sequence. Research in Veterinary Science, 89: 262-265.