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Comparison of Three Diagnostic Methods for *Theileria annulata* in Sahiwal and Friesian Cattle in Pakistan

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Abstract.- The prevalence of the blood protozoa was studied by sampling from Sahiwal and Friesian cattle, 500 each, from livestock farms in three districts of Punjab province during September 2006 to August 2007. The seasonal prevalence was higher in Friesian cattle as compared with Sahiwal cattle *i.e.* 97.6% versus 44.8 % during summer, 17.6% versus 20.8% during winter, 12.8% versus 9.6% during autumn respectively. Initial blood film examination revealed anemia, thrombocytosis, and leukocytosis and hematological findings indicated macrocytic hypochromic anemia. *Theileria annulata, Babesia bigemina* and *Babesia bovis* were detected by PCR in both Friesian and Sahiwal cattle. Out of 500 blood samples obtained from each breed of cattle 5%, Sahiwal cattle were positive in blood smears for *Theileria* as against 7% samples of Friesian cattle. Blood protozoan mixed infection with *Babesia* 1.58% was recorded in both breeds by blood smear examination during summer and spring season. On the basis of PCR 23% prevalence in Sahiwal cattle was recorded for *Theileria annulata* compared to 40.4% prevalence in Friesian cattle, while 19% prevalence for *Babesia* was recorded in both breeds during all seasons. The result of IFA test indicated 19% prevalence in Sahiwal cattle for *Theileria annulata* compared with 20% in Friesian cattle, while mixed prevalence of 8% was recorded in both breeds during summer and spring season. PCR test was concluded as more sensitive and specific as compared to IFA test.

Keywords: *Theileria, Babesia*, Friesian, Sahiwal cattle, indirect fluorescent antibody test, blood protozoa.

INTRODUCTION

Sahiwal cattle breed is mixture of strains of cattle imported from south of India and north of Pakistan and adjoining territory of Afghanistan. Protozoan diseases particularly theileriosis imposes considerable restraints on the cattle production. Tick-transmitted blood protozoan parasites of cattle are important constraints to the improvement of the livestock industry. Mortality varies from 90% in introduced exotic breeds to 5% or less in indigenous breeds. The case fatality rate of 14% has been calculated for Theileriosis at Government maintained livestock farms in Punjab, Pakistan. Mortality in fully susceptible cattle can be nearly 100 percent. In recovered cattle, chronic disease problems can occur that result in stunted growth in calves and lack of productivity in adult cattle. There are six identified *Theileria* species that infect cattle**.** **T**he two most pathogenic and economically important species of *Theileria* are *Theileria parva* and *Theileria annulata*. The two species of *Babesia* mostly prevalent in bovine are *Babesia bigemina* and *Babesia bovis*. They are responsible for mortality and losses in production that can only be guessed at, but these species are consistently recorded as the cause of major disease entities (Iriminoiu, 2000). *Theileria annulata is* transmitted transtadially by ticks of the genus *Hyalomma* while transovarian transmission of *Babesia* by ticks belonging to genus *Boophilus* species has been reported (Durrani *et al*., 2005). In endemic areas all fully susceptible cattle are affected (Moorhouse *et al.,* 2001). Neitz (1957) reported mortality varying from 30 % to 90% in exotic breeds to 5% or less in indigenous breeds by blood protozoa. Keeping in view the prevalence of tick infestation in Pakistan an attempt is made to detect blood protozoa in carriers as well as in clinical in Frisian and indigenous cattle by polymerase chain reaction test The comparative efficacy of polymerase chain reaction test, indirect fluorescent antibody test and microscopic examination was also studied.

MATERIALS AND METHODS

The prevalence of blood protozoa was studied

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by sampling from 500 Sahiwal and 500 Friesian cattle during summer, winter, autumn and spring from three districts of Punjab province in Pakistan. Screening of reference population was done on the basis of clinical sings of high fever, swelling of sub mandibular and sub scapular lymph nodes with a history of tick infestation. Blood samples were collected in EDTA coated vacutainers for identification of blood protozoa by polymerase chain reaction test (d'Oliviera *et al.,* 1995) and in without EDTA coated vacutainers for IFA test as described by Chen *et al.* (2000). Genomic DNA was isolated with the help of Gentra DNA isolation kit (PURE GENE, USA, GENTRA) according to the prescribed method. Blood smears were prepared from jugular vein and stained by Giemsa's staining technique according to the method described by Benjamin (1978). The prevalence was calculated as described by Thrusfield (1986). For IFA test antigen slides were made from the infected blood for both *Theileria* and *Babesia* and test procedure was carried out as described by Billiouw *et al.* (2002). Analysis of extracted DNA was done by agarose gel electrophoresis for which 0.8% agarose gel was used. Four primers sets were used in the present study. Primer set A consisted, of N516 (F) GTAACCTTTAAAAACGT, N517(R) GTTACGAACATGGGTTT. Primer set B consisted of 989(F) AGTTTCTGACCTATCAG, 990 (R) TTGCCTTAAACTTCCTTG (Allsopp *et al.,* 1993). Primer set C consisted of GAU9 (F) CTGTCGTACCGTTGGTTGAC, GAU10(R) CGCACGGACGGAGACCGA. Primer set D consisted of GFU5 (F) TGGCGGCGTTTATTAGTTCG, GFU6 (R) CCACGCTTGAAGCACAGGA (Guido *et al.,* 2002). PCR reaction mixture contained, 1x PCR buffer, 4 mM $MgCl_2$, 0.4 mM dNTPs, 100 pmol of forward and reverse primers, 2.5 units of Taq polymerase and 0.5 µg of total genomic DNA and final volume of reaction mixture was adjusted to 50 µl with autoclaved distilled water. The PCR reaction cycling condition involves initial denaturation at 94°C for 4 minutes and then 30 cycles each comprising of denaturation at 94°C for 30 seconds, annealing at 55 °C and extension at 72°C for 30 seconds followed by final extension at 72°C for 5 minutes. The samples were run on 1% agarose gel.

RESULTS

The disease manifestations observed clinically included high fever, swelling of sub mandibular and sub scapular lymph nodes, weakness, increased respiration and pulse and corneal opacity, anorexia, anemia, loss of condition, haemoglobinuria and rough hair coat. Neurologic sign of incoordination was also seen in weak cattle. The signs were recorded mostly in summer and spring season while in autumn and winter the only clinical manifestations of decreased milk production, incoordination, and loss of condition were observed. The clinical picture was more acute in Friesian cattle as compared to Sahiwal cattle.

Microscopic blood smear examination

Giemsa's-stained blood films contained *Theileria* piroplasms, including comma and signet-ring with diameter of 0.5-1.5 micrometer. The *Babesia* species were identified as *Babesia bigemina* with paired structure at an acute angle to each other with the dimensions of 3-3.5 x 1-1.5 micrometer while incase of *Babesia bovis* the paired structure forms were at an obtuse angle to each other with dimension of 1-1.5 X 0.5-1.0 micrometer. Variation in shapes and sizes of erythrocytes along with basophilic stippling and presence of reticulocytes were also observed (Fig.1).

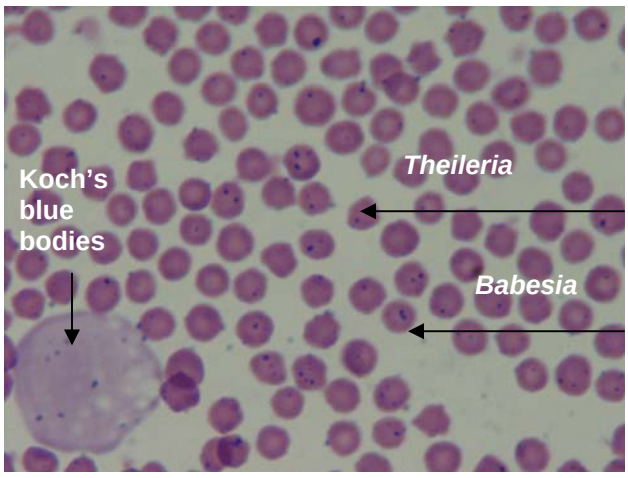

Fig. 1. Blood smears showing mixed infection of *Theileria* and *Babesia* with erythrocytic abnormalities.

The blood smear examination showed 6.8% samples positive for blood parasites. Out of these 8.823% samples were positive for *Theileria* while 12% were positive for *Babesia*. Seventy five percent of *Babesia* samples were positive for *Babesia bigemina,* while 25% were positive for *Babesia bovis*. The breed wise prevalence was higher, 7% in Friesian cattle as compared to 5% in Sahiwal cattle.

Blood parameters

Complete blood count results revealed increased mean capsular volume (MCV), mean capsular haemoglobin (MCH) values and slightly decreased mean capsular haemoglobin concentration (MCHC) value indicating persistence of macrocytic hypochromic anaemia and increased values of white blood count indicating enhanced inflammatory response in infected animals. Comparisons between infected and non infected animals showed a significant decrease in total leukocyte count from 8.41 to 5.21 x 10^3 μl^{-1}, (38%) ,eosinophils count from 0.17 to 0.11 x 10^3 μl^{-1} (35%), neutrophils count from 2.92 to 2.0 x 10^3 μl^{-1} (31.5%) lymphocytes count from 3.82 to 2.56 x 10^3 μl^{-1} (33%) and monocytes count from 0.39 to 0.30 x 10^3 μl^{-1} (23%) as shown in Table III. The result of blood parameters of total erythrocyte count, total leukocyte count, packed cell volume and hemoglobin showed significant decrease in affected Friesian cattle as compared to Sahiwal cattle.

Polymerase chain reaction PCR test

It was found out that 6 microlitre of $MgCl_2$ gave successful results than 5ul concentration for *Theileria annulata* while for *Babesia* 3ul concentration gave successful results.Primer set A amplified the expected 721 bp fragment, while primer set B amplified at 1098 bp. The primers set C and D amplified the expected 541 bp and 1124 bp band. The results showed that 41.2% samples were positive for blood parasites by PCR test. Out of these 76.9% were positive for *Theileria annulata,* and 23% were positive for *Babesia* species. The prevalence of *Babesia bigemina* was higher, 79% as compared to 21% samples were positive for *Babesia bovis*. The breed wise prevalence showed lower prevalence of 23% recorded in Sahiwal cattle compared to 40.4% for Friesian cattle. The seasonal prevalence was highest in summer, 44.8% and 97.6%, in Sahiwal and Friesian cattle respectively. During winter the prevalence was 17.6% and 20.8%, while during autumn 20% and 30.4% was recorded in Sahiwal and Friesian cattle respectively. The prevalence in spring season was 9.6% and 12.8% in Sahiwal and Friesian cattle, respectively (Table I). The overall prevalence in district Lahore was highest (13.1%) as compared to 10.8% in district Multan and 7.8% in district Rawalpindi. The species wise result in three districts is shown in Table II. The statistical analysis showed significant difference ($P<0.05$) between the prevalence of blood protozoan disease in Friesian and Sahiwal cattle during four seasons in three districts. The results also indicated that significant difference was due to prevalence of disease in summer season.

Table I.- Seasonal prevalence of *Theileria annulata* In both breeds.

S. No.	Season	Seasonal prevalence % in Friesian	Seasonal prevalence % in Sahiwal
1	Summer	97.6	44.8
2	Winter	20.8	17.6
3	Autumn	12.8	9.6
4	Spring	30.4	20

Indirect fluorescent antibody test

The results showed that 23.5% samples were positive for blood protozoa by IFA test, out of which 82.9% samples were positive for *Theileria* while 17% were positive for *Babesia*. IFA test showed higher prevalence of *Theileria*, 20% in Friesian cattle compared to 19% prevalence in Sahiwal cattle. All samples positive by IFA test were also positive with PCR test.

DISCUSSION

The seasonal prevalence of blood protozoa was highest in summer, 44.8% and 97.6%, in Sahiwal and Friesian cattle, respectively. During winter the prevalence was 17.6% and 20.8%, while during autumn 20% and 30.4% was recorded in Sahiwal and Friesian cattle respectively. The results are supported by to Flach *et al.* (1993) who had

Table II.- Seasonal prevalence of theileriosis in Sahiwal cattle and Friesian cattle from districts Rawalpindi, Lahore and Multan.

Season	Multan	Lahore	Rawalpindi	Total positive samples	Seasonal prevalence %
Sahiwal cattle					
Summer	19	28	9	56	44.8
Winter	8	10	4	22	17.6
Autumn	4	6	2	12	9.6
Spring	9	12	4	25	20
Total	**40**	**56**	**19**	**115**	
Friesian cattle					
Summer	41	43	38	122	97.6
Winter	9	10	7	26	20.8
Autumn	5	7	4	16	12.8
Spring	13	15	10	38	30.4
Total	**68**	**75**	**59**	**202**	

Table III.- Hematological observations on different post infection days of theileriosis.

Parameter	Non infected animals (*n* = 20)	0 day post infection (*n* = 80)	7 day post infection (*n* = 80)	14th day post infection (n= 80)
WBCs (10^3 μl^{-1})	8.41 ± 0.28	8.30 ± 0.30	5.57 ± 0.41***	5.21 ± 0.45***
Neutrophils (10^3 μl^{-1})	2.92 ± 0.31	2.89 ± 0.32	2.13 ± 0.34***	2.00 ± 0.37***
Lymphocytes (10^3 μl^{-1})	3.82 ± 0.15	3.10 ± 0.15	2.92 ± 0.15***	2.56 ± 0.17***
Monocytes (10^3 μl^{-1})	0.39 ± 0.02	0.36 ± 0.02	0.33 ± 0.03*	0.30 ± 0.04*
Eosinophils (10^3 μl^{-1})	0.17 ± 0.01	0.16 ± 0.01	0.14 ± 0.03**	0.11 ± 0.04**
RBCs ($10^6 \mu l^{-1}$)	9.31 ± 0.02	7.4 ± 0.20**	6.20 ± 0.23***	5.9 ± 0.26***
Hb (g/dl)	12.51 ± 0.17	8.37 ± 0.24***	8.39 ± 0.26***	8.43 ± 0.28***
PCV (%)	37.29 ± 1.7	28.08 ± 3.2***	26.08 ± 3.5***	20.08 ± 3.8***

* $P = 0.02$, ** $P = 0.01$, *** $P < 0.0001$.

reported the 76% prevalence of piroplasm in adult carriers before the disease. Anand and Ross (2001) also reported high incidences of tropical theileriosis in cross bred cattle during summer and monsoon and identified tick vector as *Hyalomma* species. Similar findings are reported by Malmqist *et al.* (2003). The prevalence of theileria recorded in Sahiwal cattle was 23% compared to 40.2% in cross bred cattle by PCR test. The results are supported by Neitz (1957), Tahir (2000) and Fukasawa *et al.* (2003). Ndungu *et al.* (2005) also reported that different cattle types were equally susceptible to the infective dose used as indicated by the length of the prepatent periods, but there was a marked difference in their development of clinical theileriosis.

The low prevalence of *Theileria annulata* in Sahiwal suggests that this breed exhibits a high level of resistance to ticks and ultimately to tick transmitted diseases. Similar findings were made by Norval (1992) who reported daily live weight gain recorded was 216 g in treated Sahiwal x Ankole while it was 185g in untreated Sahiwal x Ankole. The difference in the epidemiological status of the theileriosis reported in the present study may be due to difference in the immunity level and difference in the climates of three districts. The results are further endorsed by the findings of Flach *et al.* (1993) who reported that the probability of clinical disease in newly infected cattle was not significantly influenced by age or by the number of adult ticks, but was significantly positively associated with the cattle population on the farm. Darghouth (2004)

who reported high endemic instability was identified on the basis of low seroprevalence rates and the occurrence of the highest disease incidence in cattle at fourth Theileriosis season or more. Similar findings were made by Billiouw *et al.* (2002) who reported that absence of endemic stability was because of innate susceptibility to ECF of local Zebu cattle, virulence of the parasite and annually varying climatic factors.

The efficacy of microscopic blood smear microscopic examination, PCR test and IFA test was compared in the samples obtained from field challenges of blood protozoa in Sahiwal and Friesian cattle. The overall efficacy of microscopic blood smear examination, IFA and PCR test in field challenges of blood parasites in cattle was 6.8%, 23.5% and 41.2%, respectively. Thus it is confirmed that PCR test is more sensitive in detecting low grade of infections in carrier animals as compared with microscopic blood smear examination. Similar observations were made by d'Oliviera *et al.* (1995) and Olivier *et al.* (1999) who reported PCR, as a versatile method for the identification of multiple tick-borne infections in cattle. Similar observations were reported by Aktas *et al.* (2006). During the present study mixed infection with *Babesia* species were recorded in cross bred and Sahiwal cattle. Similar findings regarding mixed infection were made by Ziam and Benaouf (2004), Olivier *et al.* (1999), Akinboade and Dipeolu (2001), Fahrimal *et al.* (1992) and Figueroa *et al.* (1998), while detecting babesiosis in cattle.

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REFERENCES

AKINBOADE, O.A. AND DIPEOLU, O.O., 2001. Comparison of blood smear and Indirect Flourescent Antibody Test in the detection of hemoparasitic infections in trade cattle in Nigeria. *J. Vet. Sci*., **42** :326-330.

AKTAS, M., BROWN, C.G.D., HULLIGER, L., GALL, D. AND MCLEOD, W.G., 2006. A molecular survey of bovine theileria parasites among apparently healthy cattle and with a note on the distribution of ticks in eastern. *Turk. Vet. Parasitol.,* **138** : 179–185.

ALLSOPP, B.A., BAILS, H.A., CAVALIER, S.T., BISHOP, R.S., CARRINGTON, D.M. AND SOOPNER, P., 1993. Discrimination between six species of theileria using oligonucleotide probes which detect small sub unit ribosomal RNA sequences. *Parasitology,* **107**: 157-165.

ANNAND, D.F. AND ROSS, D.R., 2001. Epizootiological factors in the control of bovine theleriosis. *Aust. Vet. J*., **48:** 292-298.

BENJAMIN, M.M., 1978. *Outline of veterinary clinical Pathology*, 3rd Edition,The Iwoa State Univ. Press, Ames, Iowa, USA, pp. 7-8, 29-30.

BILLIOUW, M.L., MATAA, T., MARCOTTY, G., CHAKA, G. AND BRANDT, J., 2002. Current epidemiological status of bovine Theileriosis in Eastern Zambia. *J. Parasitol*., **56**:678-79.

CHEN, P.P., CONRAD, P.A. AND DOLAN, T.T., 2000. Detection of theileria parva in salivary glands of *Rhipicephalus appendiculatus* ticks & host animals. *Parasitol. Res*., **77**: 590-594

D'OLIVIERA, C., WEIDE, I.M.V.D., MIGUEL, I., HABELA, H., JACQUIET, P. AND JONGEJAN, F., 1995. Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *J. clin. Microbiol*., **33**: 2665–2669

DARGHOUTH, M.A., 2004 .Piroplasmids of livestock in Tunisia. *Arch. Inst. Pasteur Tunis*, **81**: 21-25.

DURRANI, A.Z., KHAN, M.S. AND KAMAL, N., 2005. Incidence of theileriosis and chemoprophylaxis with herbal medicine in cattle of union council, Jia Bagga, Lahore. *J. Anim. Pl. Sci.,* **15** :25-27.

FAHRIMAL, Y., GOFF, W.L. AND JASMER, D.P., 1992. Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction amplification of parasite DNA. *J. clin. Microbiol*., **30**: 1374-1379.

FIGUEROA, J.V., ALVAREZ, J.A., CANTO, G.J., RAMOS, J.A., MOSQUEDA, J.A., CANTO, G.J., VEGA, C.A. AND BUENING, G.M., 1998. Comparative sensitivity of two tests for the diagnosis of multiple hemoparasitic infections of cattle. *Ann. N.Y. Acad. Sci*., **23**: 791: 117-127.

FLACH, E.J., OUHELLI, H., WADDINGTON, D., OUDICH, M. AND SPOONER, P., 1993. Factors influencing the transmission and incidence of tropical theileriosis (*Theileria annulata* infection of cattle) in Morocco. *Trop. Anim. Hlth. Prod*., **25**: 75-78.

FUKASAWA, M., KIKUCHI, T., KONASHI, S., NISHIDA, S. AND YAMAGISHI, T., 2003 Assessment of criteria for improvement in *Theileria orientalis* sergenti infection tolerance. *J. Anim. Sci*., **74**: 67–72.

GUIDO, F.C.L., ANGELA, P.S., LLOYD, H.L. AND CLAUDIO, R.M., 2002. Assessment of primers designed from the small ribosomal subunit RNA for specific discrimination between *Babesia bigemina* and

B.bovis by PCR. *Cien. Anim. Brasil.,* **3**: 27-32.

IRIMINOIU, T.T., 2000. The response of Bos taurus and Bos indicus cattle types to inoculation of lymphoblastoid cell lines infected with *Theileria parva* schizonts. *Tropenmed. Parasit*., **33** : 57-62.

MALMQUIST, W.A., NYINDO, M.B.A. AND BROWN, C.D.G., 2003. Seasonal occurrence of ticks and piroplasms in domestic animals. *Trop. Anim. Hlth. Prod.,* **2**:139-145.

MOORHOUSE, P.D.S., MUSISI, F.L., MWASE, E.T. AND SNACKEN, M., 2001. The epidemiology of bovine theileriosis in Zambia: results of a longitudinal study in Southern Province. In: *Proceedings of the 4th International Symposium on Veterinary Epidemiology and Economics.* Singapore Veterinary Association, Singapore. pp. 389-391.

NDUNGU, S.G., NGUMI, P.N., MBOGO, S.K., DOLAN, T.T., MUTUGI, J.J. AND YOUNG, A.S., 2005. Some preliminary observations on the susceptibility and resistance of different cattle breeds to *Theileria parva* infection. *Onderstepoort J. Vet. Res*., **72** :7-11.

NEITZ, W.O., 1957. Theileriosis, gonderioses and cytauxzoonoses: a review. *Onderstepoort J. Vet. Res*., **27**: 275–430.

NORVAL, R.A., 1992. In: *The epidemiology of Theileriosis in Africa* (eds. B.D. Perry and A.S. Young). Academic Press, NY. pp. 481.

OLIVIER, A.E., GUBBELS, M.J., GUIDO, R., ALEXENDER, P. AND JONGEJAN, F., 1999. In: *Proceedings of the Integrated Molecular Diagnosis of* Theileria *and* Babesia *species of cattle in Italy.* STVM, 5th Biennial Conf. Society for Tropical Veterinary Medicine, pp. 130.

TAHIR, M., 2000. *To prepare vaccine against* B. microplus *and to study and evaluate the comparative efficacy of vaccines prepared from mid gut, salivary gland and whole tick in terms of antibody titers and percentage of tick rejection*. Pilot project. Livestock Experiment Station, Qadirabad, Sahiwal. Pakistan.

THRUSFIELD, M.V.,1986. Describing disease occurrence In: *Veterinary epidemiology*, 1st Edition. Butterworth & Co. Ltd. pp. 191–197.

ZIAM, H. AND BENAOUF, H., 2004. Prevalence of blood parasites in cattle from wilayates of Annaba and El Tarf east Algeria. *Arch. Inst. Pasteur Tunis*, **81**:27-30.

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