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A perspective on *Theileria equi* infections in donkeys

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Abstract

The donkey population has remained unchanged in the last two decades despite a decrease in the overall population of equids, emphasizing the usefulness of the donkey as a draught and pack animal. Piroplasmiasis in donkeys, caused by *Theileria equi* and *Babesia caballi*, has been recognized as a serious problem of major economic importance as the affected animals manifest decreased working capacity, loss of appetite, etc. In tropical countries, *T. equi* infections are more wide-spread and pathogenic than those caused by *B. caballi*. Donkeys usually remain asymptomatic carriers with positive antibody titres throughout life. Transmission of infection occurs from animal to animal through ticks such as *Hyalomma* spp. *Rhipicephalus* spp. and *Dermacentor* spp. The clinical form of the disease is diagnosed by peripheral blood smear examination, but in carrier donkeys it is very difficult to demonstrate the parasite in stained blood smears as the parasitaemia is extremely low. For diagnosis of such low grade infection or carrier animals, serological tests and DNA-based molecular diagnostic techniques, which are discussed in the present review, have become mandatory. Currently, there is no suitable pharmacotherapy available to clear the *T. equi* infection from affected donkeys, though some new drugs and drug combinations used against this disease condition have been discussed. In the present situation, there is an urgent need for international cooperation and coordination for development of sensitive molecular diagnostic tools and effective pharmacotherapies for curtailment of the disease condition. Hence, it is imperative to develop and exchange reagents and technology developed through human resource sharing in the interest of sustainability of donkey husbandry.

Key words: *Babesia caballi*, Donkey, Piroplasmiasis, *Theileria (Babesia) equi*, Tick-borne disease

Introduction

Despite the increase in mechanization throughout the world, donkeys are still well deserving of the name “*beasts of burden*.” They act as a lifeline in arid and semi-arid parts of the world by providing an economical mode of trans-

port for people and goods. This is evident by the widespread use of donkeys in rural and urban areas of Asia and Africa and parts of Central America. The overall population of equids has decreased over the last two decades; however, the population of donkeys has remained unchanged, emphasizing the value of the donkey as a draught

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and pack animal despite the mechanization of agriculture and allied fields. Besides this, the donkey is preferred over other animals due to its docile nature, disease resistance, ease to put to work and source of livelihood for the poorest of the poor. Equine piroplasmosis has become a matter of concern for donkeys not only due to its ill effects on health and work performance but also on account of their being carriers of this infection. This infection has been observed to precipitate in the event of strenuous exercise¹⁸⁾, hence carrier donkeys used as pack or draught animals become more prone to this alarming condition.

In equines, two different protozoa, *Babesia caballi* and *Theileria equi*, are known to cause the infection. The classification of *Babesia equi* into the genera *Babesia* or *Theileria* has led to controversy due to the pre-erythrocytic developmental stages as recorded in other *Theileria* species. Recent molecular-genetic techniques have demonstrated differences and similarities between these organisms. Based on these observations, *B. equi* has recently been reclassified as *T. equi*³⁹⁾ as a result of 18S rDNA sequence analysis¹⁾, which confirmed previous observations of pre-erythrocytic stages in lymphocytes^{40,51)}.

Epidemiology and etiology

Equine piroplasmosis is one of the most important tick-transmitted haemoprotozoan disease in equids (horses, donkeys, mules, and zebras). *T. equi* is a small piroplasm, whereas *B. caballi* is a large form of the parasite (Fig. 1). *T. equi* is known to be more virulent and tends to cause fulminating parasitaemia¹⁵⁾. *T. equi* infection, especially in donkeys, has been reported from tropical and subtropical countries like India^{17,30,37)}, Brazil²⁶⁾, and Arabian countries⁵⁹⁾. The distribution of this disease in Southern Africa has not been studied extensively, but probably coincides with the distribution of the tick vectors⁶⁰⁾. *B. caballi* is believed to be more widespread in Southern African countries than *T. equi*.

Ixodid ticks of the genera *Hyalomma*, *Dermacentor* and *Rhipicephalus* have been identified as vectors for the transmission of either *T. equi* or *B. caballi* protozoa to natural hosts. In tropical countries, including India, ticks of *Hyalomma* species seem to be a potential vectors for the transmission of *T. equi* to donkeys and horses. Recently, Kumar *et al.*³⁴⁾ reported that acini of *Hyalomma anatolicum anatolicum* male ticks were more infected with *T. equi* sporozoites than in female ticks, with an average of 23.95% of acini infected in male ticks compared to 13.19% in females.

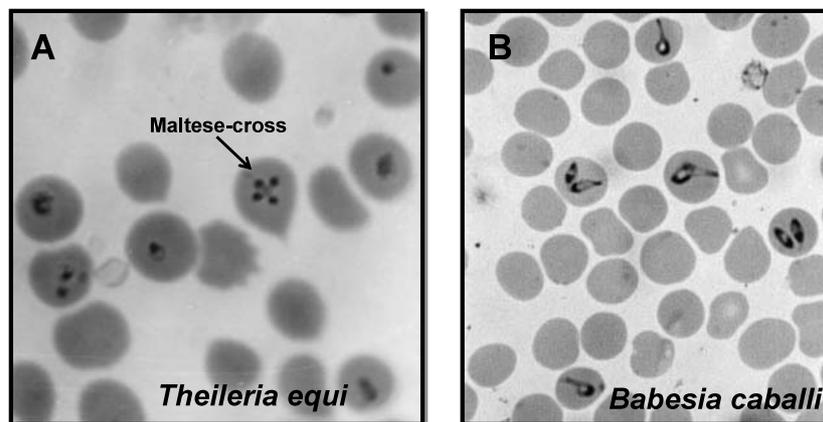


Fig. 1. Blood smear showing *Theileria equi* (A) and *Babesia caballi* (B) in erythrocytes.

Pathogenesis

T. equi and its metabolites inflict adverse effects on the erythrocytes as significant increases in the donkey's erythrocyte membrane proteins, total phospholipids and plasma malondialdehyde are observed during acute *Theileria* infection in donkeys³. These studies indicated lipid peroxidation and oxidative stress in the *T. equi*-infected erythrocytes, leading to erythrolysis, and hence decreased haemoglobin and PCV values. Furthermore, scanning electron microscopy of *T. equi*-infected donkeys confirmed this observation, as fine granulation on the erythrocyte surface and pitting of its membrane were observed².

Clinical manifestations

Donkeys generally remain asymptomatic with lower *T. equi* parasitaemia as compared to the serious clinical illness in horses due to this parasite. The disease condition starts with intermittent fever up to 40°C followed by listlessness, depression, marked thirst, inappetence, watering from the eyes, and swelling of the eyelids. Affected donkeys are constipated, passing small, hard balls of faeces covered with yellow mucus, and their health condition may degenerate. Sometimes donkeys can show colicky symptoms. An extremely large spleen is a very common symptom in the affected animals. Finally, the urine becomes dark yellow to orange or

brown, indicating the presence of haemoglobin and bile pigments as a result of severe haemolysis of the infected erythrocytes. Chronic cases of equine piroplasmosis are more common in donkeys and clinical signs are usually nonspecific, including mild appetite, poor work performance, or poor body weight gain. Splenomegaly has been observed as a common finding in affected donkeys. A recent study confirmed that newborn donkey foals were naïve at the time of foaling and that passively transferred immunity is transitory, as it wanes after 63-77 days post-foaling³⁵. Severe hepatomegaly, splenomegaly, icterus, and internal hemorrhages have also been reported in aborted foetuses⁶⁰.

Clinical pathology

Acute infection is characterized by severe leucocytosis, lymphopenia, and a high absolute neutrophil count^{32,53}. *T. equi* organisms are also observed in neutrophils and monocytes (Fig. 2) in the case of high parasitaemia, indicating phagocytosis of the infected erythrocytes³. In *Plasmodium falciparum* erythrophagocytosis and pitting of the erythrocytic membrane are also common¹. Donkeys that die of *T. equi* infection show varying degrees of emaciation, gross enlargement of the liver and spleen, and flabby kidneys⁵². Small pin-point petechial hemorrhages are also present in the liver, spleen, and on the cortical surface of the kidneys.

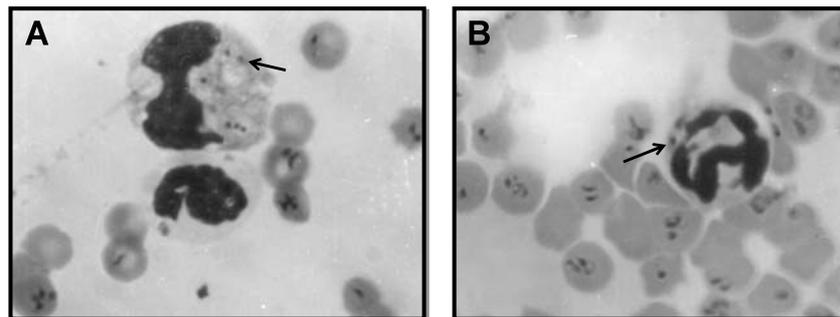


Fig. 2. Blood smear showing high parasitaemia with organisms in the monocyte (A) and neutrophil (B).

Lungs are oedematous and congested and enlarged lymph nodes are found. Microscopically, Kupffer cells exhibit deposition of haemosiderin^{14,52}.

Diagnosis

The clinical form of the disease can be confirmed by detecting *Theileria* protozoa inside the donkey's erythrocytes in blood smears stained with Romanowsky's stain (e. g., Giemsa stain). The organism may be found either singly, in pairs, or in tetrads. Formation of four daughter parasites generally called a 'Maltese-cross' is a very characteristic feature of *T. equi*. In *T. equi* infection, clinical parasitaemia may exceed 20%, whereas 1-5% parasitaemia is more commonly observed in field conditions. It is very difficult to demonstrate the parasite in latent carrier donkeys as the parasitaemia is extremely low. Hence, serological and other tests are used to detect the carrier status.

Serological tests

Complement Fixation Test: The complement fixation test (CFT) is one of the oldest serological tests and was used for the first time by Hirato *et al.*¹⁹ for diagnosis of *B. caballi* antibodies in horses. Since it was the most specific and sensitive diagnostic test available at that time for detection of *T. equi* antibodies, the Office International des Epizooties (OIE) prescribed this test along with IFAT as a qualifying test for international trade (Office International des Epizooties, 2000)⁴¹. Donkey and zebra sera are known to show anti-complementary activity in the CFT²⁴. Kumar *et al.*³⁰ reported that the CFT for detecting *T. equi* antibodies should be standardized separately for donkeys as the condition optimized for horse samples failed for donkey samples. They advocated that the donkey serum should be inactivated at 59°C for 30 min instead of the conventional 56°C for 30 min. This practice reduced the nonspecific fixing of complement by the

donkey serum. Considering all these inherent drawbacks of the CFT, this test has now been removed from the prescribed list of tests recommended for *T. equi* infection by the OIE for international trade (2004)⁴² and is described as an alternate test.

ELISA: Many versions of ELISA have also been standardized for detecting *T. equi* antibodies in carrier donkeys. Among these, Dot-ELISA³⁰, serial dilution ELISA and single dilution ELISA³³ are the most important. Dot-ELISA can detect *T. equi* antibodies in experimentally infected donkeys as early as 3-6 days post-infection and remains positive, showing a high antibody titer, at least until 90 days. Serial dilution ELISA is more time consuming and requires large quantities of antigen, conjugate or reagent as compared to single-dilution ELISA when the end titre of the sample is to be determined^{33,38,54}. ELISA based on a purified recombinant antigen is more specific, and sensitive and gives no cross-reaction. The prime prerequisite for the development of recombinant ELISA is the identification of the antigen that is highly immunodominant and is able to produce strong and lasting antibodies in the natural host during the course of infection. Equine merozoite antigen-1 (EMA-1, 34 kDa), a *T. equi* erythrocyte-stage protein, possesses an epitope that has been shown to be both immunodominant and conserved worldwide^{25,28}. Equine merozoite antigen-2 (EMA-2) shares 52% amino acid identity with EMA-1²⁹. Recently Knowles and associates²⁷ defined a monoclonal antibody-based competitive inhibition ELISA (CI ELISA) for diagnosis of *T. equi* antibodies. They prepared monoclonal antibodies (Mab 36/133.97) against *T. equi* merozoite antigen EMA-1, confirmed the superior sensitivity of the CI ELISA to CFT, and found CI ELISA to be more sensitive and specific. Recently, the OIE also recommended the CI ELISA as a test for international trade of horses⁴².

Other serological tests, like the capillary tube agglutination test^{30,37} and countercurrent immunoelectrophoresis test¹², are also used and standardized for diagnosis of *T. equi* infection in donkeys,

but these are less sensitive and specific.

PCR and DNA probes: Routine serological tests can indirectly detect the parasitic antigen. Hence, direct detection of the specific parasitic DNA by means of DNA probes and the polymerase chain reaction are desirable in valuable animals or animals in disease-free zones. Posnett *et al.*⁴⁶⁾ and Posnett and Ambrosio^{44,45)} reported DNA probes for diagnostic tests capable of detecting parasitaemia levels as low as 0.0028% (*T. equi*) and 0.0016% (*B. caballi*) in blood samples. PCR-based diagnostic tests have been standardized for the detection of *T. equi* and *B. caballi* parasitic DNA in the blood^{5,47)}. PCR methods have also been used to detect *T. equi* infection in the tick-vectors *Boophilus microplus* and *Dermacentor nuttalli*^{6,7)}.

Treatment and Control

Chemotherapy: A variety of drugs are used for the treatment of *T. equi* infection in donkeys. Most of the drugs improve the clinical signs but are unable to completely eliminate the infection from the body. At the same time, complete eradication is seldom mandatory in endemic areas, but lack of it entails the risk of serious relapse of the disease condition in the event of physiologically stressful conditions. Tetracycline, like chlortetracycline hydrochloride (Aureomycin[®], Ledera Laboratories) and oxytetracycline hydrochloride (Terramycin[®], Pfizer), is effective only against *T. equi* when given intravenously daily for two or more days at a dosage rate of at least 5.5 mg/kg body weight²³⁾. The time interval between doses seems to be important. Four intramuscular doses of imidocarb dipropionate (Imizol[®] Burroughs Wellcome and Co., U.K.), 72 hr apart at 4.0 mg/kg body weight, cleared *T. equi* from horses but not from infected donkeys, which died even after the treatment^{8,13)}. Two-dose therapy using imidocarb, with a 48 hr interval at 5 mg/ml, was found to be quite effective in bringing about clinical recovery in infected donkeys⁵³⁾.

Dennig *et al.*¹¹⁾ reported that diminazene diacetate (Berenil[®] Hoechst Pharmaceuticals,

Ltd.) was the only drug that was successful against mild-to-moderate *T. equi* infection in horses and donkeys, but that it was not effective against acute infection. Furthermore, diminazene diacetate was effective in eliminating *B. caballi* infection but not the *T. equi* parasite^{36,57)}. Singh *et al.*⁵³⁾ used diminazene diacetate for donkeys infected with *T. equi* at a dose rate of 12.0 mg/kg body weight, with two intramuscular injections 24 hr apart, and observed that parasitaemia only declined for two to three days after treatment. Copper glycinate has proved successful in treating clinical cases due to *Babesia bigemina* infection in cattle⁴²⁾. However, similar trials in splenectomised and non-splenectomised donkeys infected with *T. equi* and treated with intravenous injection of copper glycinate at 1.5 mg/kg, using two injections 24 hr apart, proved unsuccessful in reducing fulminant parasitaemia³¹⁾.

Encouraged by the high therapeutic efficacy of artemisinin (qinghaosu) derivatives such as artesunate, arteether, and artemeter against multiple-drug-resistant cases of falciparum malaria^{10,43,58)} and buparvaquone against tropical bovine theileriosis caused by *Theileria annulata*⁶¹⁾, we tested the therapeutic efficacy of these drugs (alone or in combination) against *T. equi*-infected splenectomised donkeys³³⁾. Animals treated with imidocarb and arteether+buparvaquone in combination were able to clear the parasite from the blood circulation at 2-5 days post-treatment (PT), but recrudescence (in both these groups) of *T. equi* was observed at 55-58 days PT.

Immunological: Very scanty efforts have been made with regard to development of a suitable and potent vaccine for the control of equine piroplasmosis in horses and donkeys. Some efforts towards vaccinological control have been made using a crude *T. equi* immunogen in donkeys. Singh *et al.*⁵³⁾ immunized donkeys with a *T. equi*-infected erythrocyte lysate, followed by boosted inoculation. Immunized donkeys survived after challenge infection, but become carriers of *T. equi*. Salem *et al.*⁴⁹⁾ also tried a crude vaccine on donkeys and reported protection upon challenge. Kumar *et al.*³²⁾

immunized donkeys with an immunogen, each dose of which contained a lysate of 2×10^{10} parasitized erythrocytes. The immunized donkeys survived after challenge infection (1×10^{11} parasitized erythrocytes), and simultaneously very high humoral and cell-mediated immune responses were mounted by the immunogen in the immunized donkeys. They opined that the *T. equi* crude immunogen could elicit a strong, protective immune response against *T. equi* infection. They also identified immunodominant polypeptides (112, 45, 33 and 18 kDa) against which strong immune responses were directed³². More vaccinological experiments are required in the future to come create a potent, safe, and specific vaccine.

The way forward

There is little doubt that the donkey and mule have been not only the most used, but also the most abused animals in history. The donkey is hardier than the horse, and can survive with much less attention, derive sustenance from poor quality food, and tolerate considerable heat and dehydration. This makes it a suitable animal for harsh environments and difficult working conditions. In rural areas, donkeys are generally highly appreciated, but many people living in towns and cities seem to have a poor view of the donkey. The vast majority of donkeys are used for the same types of work that they have been doing for 6,000 years. The donkey plays a vital role in the socioeconomic life of the poor farming population; hence the attention of researchers is needed so as to improve its health condition. Donkeys act as a carrier of equine piroplasms, which affect their health and work performance. The disease continues to have a significant impact on international trade and poses a potential threat to disease-free areas. The preliminary diagnosis begins with a descriptive history and subsequent blood smear examination. In the last decade, remarkable and significant advances have been made in the field of molecular diagnosis, including the development of recombinant

-based diagnostic assays, PCR and DNA probes. These modern molecular diagnostic techniques help researchers in identifying the disease condition even if the parasitaemia or antibody titer is quite low.

There are still many grey areas in genomic research that could be targets for the development of new diagnostic techniques and identifying novel vaccine targets and drug molecules. Rhoptry proteins have been reported to be involved in the parasitic invasion of erythrocytes⁵⁰ and might be suitable targets. Ikadai *et al.*^{21,22} identified BC48 (48 kDa) as a rhoptry protein in *B. caballi* and developed an ELISA test using the GST-fusion protein. The ELISA was able to differentiate very clearly between *B. caballi*-infected horse sera and *T. equi*-infected horse sera and noninfected normal horse sera. Rhoptry-associated proteins have also been identified in other *Babesia* species^{55,56,62} and successfully used in ELISA as diagnostic targets^{9,16}. Such information is lacking for *T. equi*, and rhoptry-associated proteins may be potential genomic and serological targets for the development of novel, specific diagnostic techniques. Lastly, international cooperation and coordination of future research, in terms of exchange of reagents and technologies, validation of tests, human resource sharing, and sustainability, are a must in the interest of development of animal husbandry.

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