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Parasitological and molecular confirmation of *Theileria equi* Infection with clinical management in a Kathiyawadi horse

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Abstract
Theileria equi (*Babesia equi*) and *Babesia caballi* are the causative agents of equine piroplasmosis, a hemoprotozoan disease spread by ticks. *T. equi* is more pathogenic than *B. caballi* and can establish lifelong latent infections in recovered animals, making eradication challenging. Transmission occurs primarily through ixodid ticks such as *Hyalomma anatolicum*, *Rhipicephalus microplus*, and *Haemaphysalis* spp. The majority of early diagnosis of *T. equi* in India were made via traditional blood smear examination, however clinical cases are regularly recorded. The horse in this study had an acute *T. equi* infection and showed clinical symptoms such as fever, anaemia, oedema, haemoglobinuria, icterus, depression, and anorexia. The animal had icteric mucous membranes, a fever (104°F), and ventral abdominal oedema, according thorough physical and clinical investigations. The results of a thin blood smear made from capillary blood drawn from the tip of the ear showed that *Babesia* spp. A 595 bp amplification product was obtained via PCR targeting the *T. equi*-specific EMA1 gene, which was used to confirm the species' identity and the molecular presence of *T. equi*. Diminazene aceturate (Berenil) was given intramuscularly once to the afflected horse at a dose of 5 mg/kg. Over the course of three days, the patient received supportive care in the form of vitamin B-complex, meloxicam, Ringer's lactate, intravenous dextrose normal saline, and oral hematinics.

Keywords: *Theileria equi*, *Babesia equi*, PCR, equine piroplasmosis, diminazine aceturate

Introduction
The intra-erythrocytic protozoa *Babesia equi* and *Babesia caballi*, the latter of which has been reclassified as *Theileria equi* (*Babesia equi*), are the causes of the endemic, tick-borne hemoprotozoan disease known as equine babesiosis (Alhassan *et al*., 2007; Bhojani *et al*., 2021) [1, 6]. The incidence of these parasites is tightly linked to the spread of tick vectors, and they are found around the world (Kumar 2007; Motloang *et al*., 2008; Kumar *et al*., 2020) [12, 18, 13].

Equine piroplasmosis can present with a variety of clinical symptoms, including as fever, anaemia, icterus, hepatomegaly, ventral oedema, haemoglobinuria, intravascular haemolysis, and, in extreme situations, mortality (Uilenberg, 2006) [25]. It is generally accepted that carrier animals are the main way that infections persist and spread (Constable *et al*., 2017; Bhojani *et al*., 2023) [8, 7]. The virulence of the infection and the sensitivity of the host both affect the severity of the disease. Severe clinical indications are more likely to appear in horses under physiological stress or those transported from tick-free to tick-endemic regions (Onyiche *et al*., 2019) [19].

Equine babesiosis is spread biologically by a number of tick species, including those belonging to the genera *Boophilus*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Battsetseg *et al*., 2002) [2]. Clinical symptoms and microscopic analysis of Giemsa-stained thin blood smears are the main methods used to diagnose acute infections. However, low parasitemia can make it difficult to diagnose chronic or subclinical instances; piroplasms must be detected by competent microscopy (Hodgson, 2002) [11]. Furthermore, mixed infections have been recorded often, and it is challenging to distinguish between *T. equi* and *B. caballi* infections based just on clinical indicators (Xu *et al*., 2003; Alhassan *et al*., 2007) [27, 1]. According to Salib *et al*. (2013) [20], anti-theilerial medications can be used in conjunction with supportive care to treat equine piroplasmosis.

Nevertheless, diseased horses may still serve as reservoirs for both *Babesia* species even after they have recovered clinically (de Waal, 1992; Mavadiya *et al*., 2023) [9, 17]. The current study was conducted to evaluate the clinical manifestation and treatment of equine piroplasmosis in horses from the Saurashtra region of Gujarat, India, in light of these worries (Bharai, 2018; Bharai *et al*., 2020) [4, 5].

History and Diagnosis

When a seven-year-old Kathiawadi horse was brought for treatment, it had icterus, haemoglobinuria, anaemia, dependant oedema, fever, anorexia, and sadness. Ventral abdominal oedema, icteric mucous membranes, and a high body temperature of 104°F were discovered during a thorough physical and clinical examination. A tiny smear of capillary blood taken from the tip of the ear was used to diagnose an infection with *Babesia* spp. Giemsa was used to stain the smear, and oil immersion microscopy was used to examine it. Small, paired pyriform parasites seen in red blood cells were used to identify *Babesia equi* (*Theileria equi*).

Materials and Methods

a) Parasitological Examination

After the smear from blood samples were prepared, thin blood smears were processed by using Giemsa stain. Methanol was used to fix the smears, and then they were stained for 40-45 minutes by using a diluted Giemsa stain (1:20). After 45 minutes slides were cleaned under running tape water for 2-3 minutes and allowed to air dry. Then, using an oil immersion lens set to 100x magnification, the stained smears were examined. In order to assess the presence of *Theileria* spp. parasites, at least eight fields were chosen for observation along the smear's edge. The parasites were identified by their distinctive morphological characteristics, which helped to differentiate them from potential staining artifacts.

b) DNA extraction and PCR amplification

Using the QIAamp DNA Blood Mini Kit (Qiagen, Germany), genomic DNA was extracted from whole blood in accordance with the manufacturer's instructions. 200 µL of elution buffer was used to elute the DNA, which was then kept at-20 °C until it was needed. *Theileria equi* EMA1 and RAP genes were amplified using Polymerase Chain Reaction (PCR) for molecular validation.

Two distinct PCR reactions were conducted utilising certain primer pairs in order to identify the species of *Babesia equi* (*Theileria equi*) and *Babesia caballi*. The primer pair BcF (5′-AGACCTTATTGGCTGCC-3′) and BcR (5′-CGCGAGTCACGTTCTTCT-3′) targeted a 414 bp fragment of the *B. caballi* RAP-1 gene, whereas the primer pair BeF (5′-AAGCAGTCCGAGGAGCA-3′) and BeR (5′-CTGGGAAGGTGCTGTTG-3′) amplify a 595 bp fragment of the *T. equi* EMA-1 gene (Kumar *et al*., 2021). Each 25 µL PCR reaction comprised 12.5 µL of 2× DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Lithuania), which contains optimised quantities of Taq DNA polymerase, reaction buffer, $MgCl_2$, and dNTPs. Moreover, 4 µL of template DNA and 1 µL of each forward and reverse primer (10 µM) were added. The thermal cycling conditions were as follows: a 5-minute initial denaturation at 95 °C, 35 cycles of denaturation at 94 °C for 30 seconds, 35 seconds of annealing at 58 °C for *T. equi* and 52 °C for *B. caballi*, one-minute extension at 72 °C, and a final extension at 72 °C for Ten minutes. PCR amplicons were observed under UV light using a gel documentation system (Gel Doc™ XR +, Bio-Rad, USA) after being resolved on a 1% agarose gel stained with ethidium bromide.

c) Therapeutic management

After the diagnosis was established, the horse injected with single intramuscular injection of 5 mg/kg of diminazene aceturate (Nilbery). Supportive care consisted of oral hematinics (Sarkoferrol Vet) for three days in a row, intravenous injection of Ringer's lactate and dextrose normal saline, meloxicam (Melonex) at 0.2 mg/kg, and vitamin B-complex (Tribivet). The animal's vital values returned to normal following three days of treatment, and a gradual improvement in clinical status was seen.

Results and Discussion

a) Animals

The kathiawadi horse's clinical state was critical and worrisome; it was marked by high *Babesia* spp. parasitemia and significantly low haemoglobin levels, as shown by the pale conjunctival mucosa (Fig. 1A). The animal's initial reaction to therapy was gradual because of the severity of the infection. The horse exhibit a noticeable clinical improvement after the entire treatment plan was finished. A good recovery was shown by the post-treatment evaluation, which showed no *Babesia* spp. parasites in Giemsa-stained blood smears.

b) Microscopic Examination

In the stained blood smears, *Babesia* species were recognised as tiny piroplasms that formed distinctive maltese cross structures (Fig. 1B, 1C). This is a morphological property that sets *Babesia equi* (*Theileria equi*) apart from *Babesia caballi*, which is distinguished by its bigger piroplasm forms. Additionally, *T. equi* organisms were seen alone, in pairs, and sporadically inside neutrophils. There are several documented clinical examples of *T. equi*-caused equine piroplasmosis, which is endemic in India (Kumar *et al*., 2013) (14). Previous reports of *T. equi* infections in India were based mostly on morphological identification using stained blood smears; molecular confirmation was not used (Sharma *et al*., 1982) (22). The goal of the current investigation was to molecularly characterise *T. equi* field strains that were obtained from horses that were clinically afflicted. By examining Giemsa-stained blood smears under a microscope, where *Babesia* spp. were easily apparent, the *B. equi* infection in the present instances was verified. Compared to *B. caballi*, *T. equi* has a very different morphology (Kumar and Kumar, 2007) (12). About 2.0 × 1.0 µm in size, *T. equi* resembles tiny piroplasms that frequently take the form of ovoid, spherical, or Maltese cross forms. *B. caballi*, on the other hand, is larger (2.5-4.0 × 1.5-3.0 µm) and usually found as a single or paired round, oval, or pyriform organism (Kumar and Kumar, 2007) (12). The Maltese cross form of *T. equi* was consistently found in all clinical samples that were investigated, along with other dividing forms of the parasite.

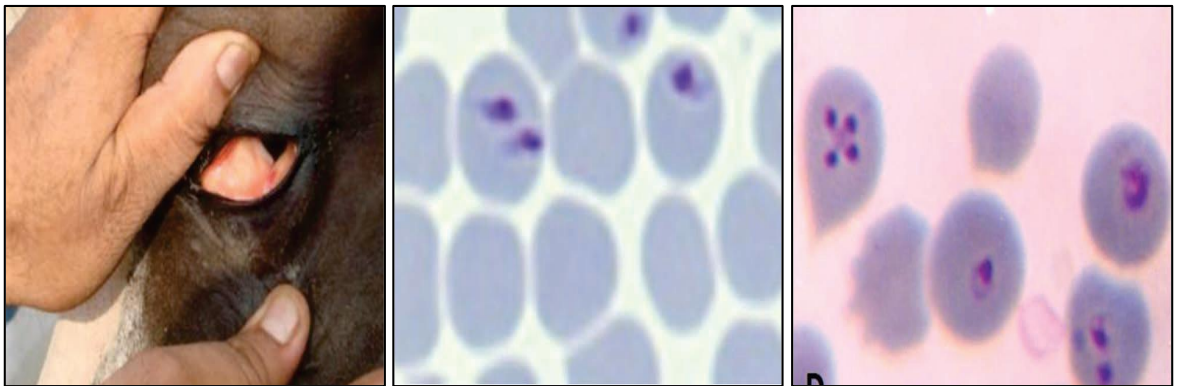

Fig 1: Photographs showing clinical sings in a *Theileria* spp. infected horses and various developmental stages in the blood smears.

PCR Amplification

For the purpose of amplifying EMA-1 and RAP gene segments unique to *T. equi*, sense and antisense PCR primers were created. The following primer sequences were found: 5′-AAGCAGTCCGAGGAGCA-3′ (forward) and 5′-CTGGGAAGGTGCTGTTG-3′ (reverse) for EMA-1; 5′-AGACCTTATTGGCTGCC-3′ (forward) and 5′-CGCGAGTCACGTTCTTCT-3′ (reverse) for RAP. The EMA-1 and RAP genes produced DNA fragments of 595 bp and 414 bp, respectively, through PCR amplification. A 595 bp fragment belonging to the EMA-1 gene was effectively amplified in the DNA samples taken from clinically infected horses, demonstrating the existence of DNA specific to *T. equi*. The 414 bp RAP gene fragment, however, was not amplified from the identical DNA samples, suggesting that it was either absent or present at undetectable quantities at the specified PCR conditions (Vidhyalakshmi *et al*., 2018; Sanjeev *et al*., 2020) [26, 21].

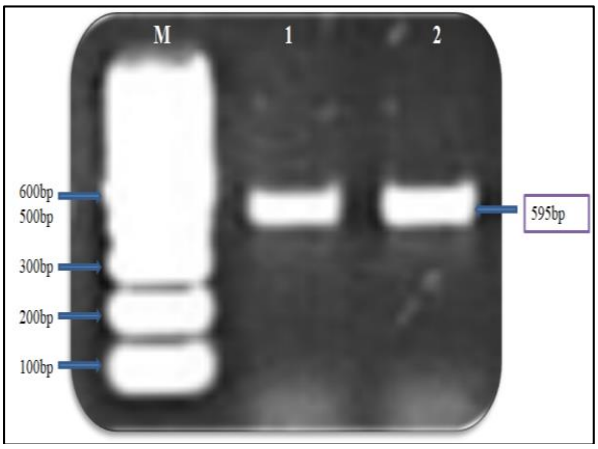

Fig 2: *T. equi* specific EMA-1 gene amplification in DNA isolated from blood samples collected from clinically infected horses. Lane: M-100bp ladder; 1,2 PCR amplification in DNA isolated clinical cases at 595 bp.

It was confirmed that the distinct EMA-1 gene sequence was present in the clinical samples by the effective amplification of *Theileria equi*-specific DNA in the PCR process. In line with other findings, Bhagwan *et al*. (2015) [3] used PCR to identify *T. equi* nuclear material in DNA isolated from tick and blood samples. The primers employed in this investigation were made especially to target *T. equi's* EMA-1 gene, which is renowned for its great sensitivity and can identify as little as 2.5 ng/µL of particular DNA (Kumar *et al*., 2020; Maharana *et al*., 2024) [13, 16].

In summary, our study effectively recorded clinical instances of *Theileria equi* infection in kathiyawadi horses and used molecular diagnostics to confirm the parasite's existence. By focussing on the EMA-1 gene and using PCR amplification and DNA extraction, an Indian strain of *T. equi* was discovered. The findings demonstrated the value of molecular techniques for precise diagnosis and strain identification by validating the genomic presence of *T. equi* in clinically infected horses.

Both types of Babesia carry the potential to infect almost all horses. Animals that survive the infection frequently develop into chronic carriers, and older animals are typically more severely impacted than younger ones (Maharana *et al*., 2024) [16]. Generalised depression, anorexia, incoordination, mucous nasal discharge, eyelid puffiness, and frequent recumbency are typical clinical symptoms. *Babesia caballi* is linked to more chronic fever and anaemia, but *Theileria equi* is thought to be more harmful among the two species (Soulsby, 1982) [23]. Depression, fever, anorexia, and jaundice were the most noticeable clinical symptoms in this instance. Using both molecular diagnostics and blood smear analysis, the presence of *T. equi* was verified. The preferred medication for removing the carrier state, diminazene aceturate (Nilbery), was administered intramuscularly once at a dose of 5 mg/kg as part of the therapy regimen. Among the supportive therapies were intravenous Ringer's lactate and dextrose normal saline, meloxicam (Melonex) at 0.2 mg/kg, vitamin B complex (Tribivet), and oral hematinics (Sarkoferrol Vet) given for three days in a row (Sudan *et al*., 2013; Dodiya *et al*., 2022) [24, 10].

Conclusion

Our research effectively documented clinical instances of *T. equi* infection in Kathiyawadi horses and used molecular diagnostics to confirm the parasite's existence. By focusing on the EMA-1 gene and employing PCR amplification and DNA extraction, an Indian strain of *T. equi* was discovered. The results demonstrated the value of molecular techniques for precise diagnosis and strain identification by verifying the genomic presence of *T. equi* in clinically infected horses. A single intramuscular dose of diminazene aceturate (Nilbery), the medication selected to eradicate the carrier state, was administered as part of the treatment regimen at a dose of 5 mg/kg. Intravenous Ringer's lactate and dextrose normal saline, vitamin B complex (Tribivet), meloxicam (Melonex) at 0.2 mg/kg, and oral hematinics (Sarkoferrol Vet) were all part of the supportive therapy.

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