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Review

Diagnostic methods used for the detection of *Theileria equi*: review of the last decade

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Abstract: *Theileria equi* is one of the aetiological agents responsible for EP and is transmitted by ticks to horses, mules, donkeys and zebras. Clinical signs are often nonspecific and can easily be confused with other pathologies. Although acute, sub-acute and chronic forms have been described, the most common situation in equines is that of asymptomatic carrier, characterized by undetectable or extremely low parasitaemia and lack of clinical signs. Identification of the parasitic agent, as well as the immunity acquired as a result of infection can be done by direct and indirect methods such as molecular and serological methods. This study aims to identify the most commonly used diagnostic methods of EP with the highest specificity and sensitivity and the fewest limitations. In order to achieve the aim of this study, a systematic database search was carried out, resulting, after a preliminary selection, in a total of 97 publications considered eligible. It was concluded that molecular diagnostic methods can overcome many of the limitations of traditional methods and are essential to identify and distinguish genotypes of *T. equi*. Nonmolecular diagnostic methods may lack sensitivity and specificity, but they are still widely used and useful to support clinical and epidemiological research.

Keywords: *Theileria equi*, equine piroplasmosis, PCR, cELISA, blood smear.

1. Introduction

Equine piroplasmosis (EP) is a disease of Equidae caused by *Theileria equi*, *Theileria haneyi* and *Babesia caballi* [1,2] transmitted by ticks to horses, mules, donkeys and zebras. Infected animals can remain carriers for long periods of time and act as sources of infection for tick vectors. Introducing carrier animals into an area where tick vectors exist can lead to an epizootic spread of the disease. Transplacental transmission of *T. equi* from carrier mares to their foetuses has also been shown [3]. Although acute, sub-acute and chronic forms have been described, the most common situation in equines is that of asymptomatic carriers. In the chronic form, animals show a dry symptomatology such as decreased exercise tolerance, while the carrier stage is characterized by undetectable or extremely low parasitaemia and lack of clinical signs [4].

Identification of the parasitic agent can be done by direct methods, blood or stained organ smears during the acute phase of the disease and by molecular and serological methods in carrier animals, low parasite burden makes detection extremely difficult [5].

The sensitivity of microscopic examination of blood smears and smears of lymph node needle aspirates is low, so that false negative results are regularly observed [6]. The most important feature is that this method is only useful in detecting infected erythrocytes in the acute phase of the disease.

Several serological tests have been developed to increase the sensitivity of the diagnosis, especially in those carrier horses that show no clinical signs. These tests include the complement fixation test (CFT), indirect immunofluorescence assay (IFA), Western blot (WB) and competitive enzyme-linked immunosorbent assay (cELISA) [5].

The CFT test depends on complement activation during the specific antibody-antigen interaction. Infected horses seroconvert on CFT approximately 8 to 11 days after infection, and titters begin to decline at 2 to 3 months [7,8]. CFT is a very specific test, but is

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not sensitive in chronic or inapparent phases of infection, mainly because some antibodies produced during these phases of infection do not bind complement [9].

IFAT is thought to be more sensitive than CFT during chronic infection. However, the need to dilute serum to improve specificity in IFAT performance reduces sensitivity. IFAT is often used as an adjuvant test to help analyse CFT results [5]. Sensitivity and specificity of CFT and IFAT for *T. equi* were reported differently, thus a sensitivity between 47% and 63% was reported for CFT and 89-96.6% for IFAT, and specificity was between 94-96% for both [10].

A method recommended and approved by the OIE for international testing for equine piroplasmosis is cELISA, which is considered the most sensitive test for the detection of chronic or inapparent *T. equi* [11,12].

The cELISA technique described by commercial kit manufacturers involves binding of primary monoclonal antibodies to antigen-coated plate, binding detected using horseradish secondary peroxidase (HRP). The presence of the HRP marker of the secondary antibody is quantified by the addition of an enzyme substrate and subsequent development of the color product. A poorly developed colour is due to inhibition of binding of the primary monoclonal antibody to the solid phase antigen and indicates the presence of *T. equi* antibody in the serum sample. [12].

Polymerase Chain Reaction (PCR) is a widely used method to rapidly make millions or billions of copies (full or partial copies) of a given DNA sample. In conventional PCR, after amplification, PCR products or amplicons are run on agarose or PAGE gels to detect the presence or absence of DNA amplification. But in Real Time PCR, amplification is monitored after each PCR cycle. Nested PCR is a modification of PCR that was designed to improve sensitivity and specificity and involves the use of two primer sets and two successive PCR reactions [13,14]. The basic principle of multiplex PCR is the same as that of conventional PCR, except that multiple primer pairs are required in the same reaction. Primers can be specifically combined with the corresponding DNA template, and more than one DNA fragment will be amplified simultaneously in a single reaction [15].

The PCR technique is one of the OIE recommended methods for the diagnosis ofEP, suitable for: infection-free equine population, infection-free individual animal, contribution to eradication measures, confirmation of clinical cases, prevalence of infection-surveillance. Several PCR diagnostic protocols are currently available, some of which are recommended by the World Organisation for Animal Health [5].

Unlike molecular diagnostic methods, serological tests have limited sensitivity and specificity. PCR help to identify asymptomatic carriers and can identify a low parasitaemia of up to 0.017 % for *T. equi* [16,17]. Application of PCR assays, targeting EMA-1 gene, BC-48 gene and 18S ribosomal RNA (rRNA) gene, demonstrated a higher level of analytical sensitivity and specificity than serological and microscopic detection.

2. Materials and Methods

To achieve the aim of this study, a systematic multi-stage search of Pubmed and Science Direct databases was conducted to identify all eligible studies.

The keywords "equine piroplasmosis", "PCR", "molecular diagnosis", "Theileria equi", "blood smear", "cELISA" were entered. Articles were selected from the period 2012 to 2022 and had as subjects the diagnosis by molecular methods and description of new protocols for molecular diagnosis of *T. equi*, identification of parasites by direct microscopy and serological methods.

The key terms "equine" and "equine piroplasmosis" allowed the identification of studies in both horses and donkeys.

After selecting papers based on titles and abstracts, studies were further analysed by detailed examination of the full text. Articles that were included in the study had to meet all of the following criteria:

(i) original research articles based on molecular diagnostic techniques, direct microscopy and serological methods;

(ii) study conducted between 2012 and 2022;

(iii) the diagnostic method must be clearly specified.

The research resulted in 478 articles, which were subsequently checked to determine whether they met all the proposed criteria as well as to eliminate duplicates. After a preliminary screening of the studies performed a total of 97 publications were considered eligible.

From 266 articles results following the introduction of the keywords molecular diagnostic and PCR, 32 were aimed at the determination of EP by molecular methods and 4 articles presenting the development and validation of a new molecular diagnostic protocol for EP (fig. 1).

Regarding the identification of parasite species by direct microscopy, a total of 7 articles were identified.

Serological diagnostic methods are frequently used in EP diagnosis, thus following the primary search in the two databases a total of 202 published articles were identified and based on the selection criteria 54 articles were considered eligible (fig. 2).

3. Results

One of the main objectives of this analysis was to identify the most commonly used diagnostic method with the highest specificity and sensitivity and the fewest limitations.

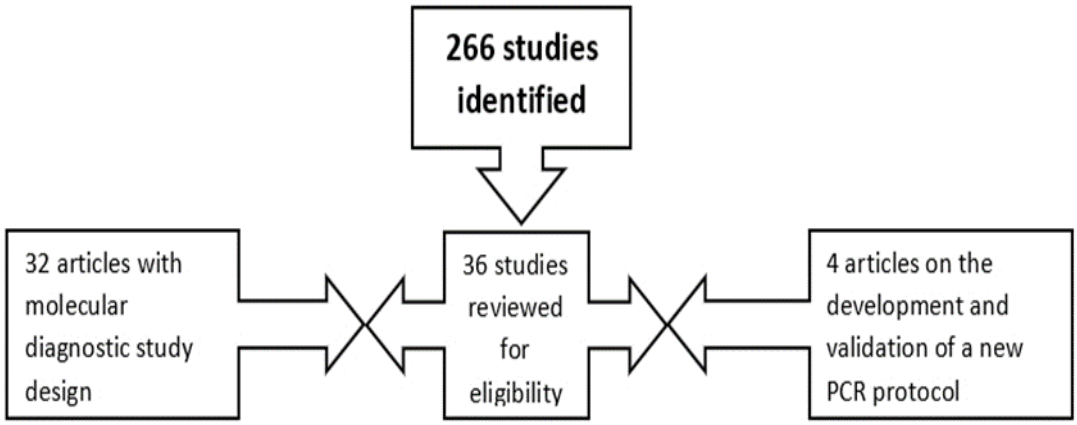

Figure 1. Selection process of studies using molecular diagnostic methods

Of the 36 articles studied, 14 authors used nested PCR, 11 conventional PCR, 6 Real Time PCR and 5 Multiplex PCR as molecular diagnostic methods.

Following the analysis of the 4 articles based on the development and validation of a new molecular di-agnostic protocol, it was concluded that 2 articles aimed to develop a new Real Time PCR protocol, one conventional PCR and one nested PCR.

The primers and probes, in the case of Real Time PCR, used were selected by the authors according to the targeted genes, namely 18S rRNA as well as BC48 (*B. caballi*) and EMA-1 (*T. equi*) genes.

Table 1. Primers and probe used in the articles studied

No	Primers	Probe	Reference
1	GTAATTCCAGCTCCAATAG AAAGTCCCTCTAAGAAGC	TTCGTTGACTGCGCTTGGCG CTAAGAAGCGGAAATGAAA	[18]
2	TCGAAGACGATCAGATACCGTCG	TGCCTTAAACTTCCTTGCGAT	[19]
3	GAAAYTGCGAATGGCTCATTAM CACCGGATCACTCGATCGGTAGG	GGATAACCGTGSTAATTSTAGGGC GTGTGTACAAAGGGCAGGGACG	[19]
4	5′- TCGAAGACGATCAGATACCGTCG-3′	5′- TGCCTTAAACTTCCTTGCGAT-3′	[20]

5	GCATCCATTGCCATTTCGAG TGCGCCATAGACGGAGAAGC AATGTTGAGCAAGTCCTTCG TTAGTAGAACAAAGCAACGGC		[21]
6	5' GGT TGA TCC TGC CAG TAG T-3′ 5' TTG CGA CCA TAC TCC CCC CA-3′		[22]
7	5′- GTCTTGTAATTGGAATGATGG-3′ 5′-TAGTTTATGGTTAGGACTACG-3′		[23]
8	5′-TCGACTTCCAGTTGGAGTCC-3′ 5′-AGCTCGACCCACTTAT CACC-3′ 5′- ATTGACCACGTCACCAT CGA-3′ 5′-GTCCTTCTTGAGAACGAGGT-3′		[24]
9	5′- CTGACTACAAGGTYGTATAC-3′ 5′-TGTCGTCACTTAGTAAAATAGA -3′	TeEMA1-P 6-FAM- TTCTCCGTCTATGGCGCA- MGBNFQ	[25]
10	5'- AAGCCATGCATGTCTAAGTATAAGCTTTT-3' 5'- GAATAATTCACCGGATCACTCG-3'		[26]
11	5‟- TTCGTTGACTGCGCTTGGCG-3‟ 5‟-CTAAGAAGCGGAAATGAAA-3‟		[27]
12	5′- TCGAAGACGATCAGATACCGTCG-3′, 5′-CTCGTT CATGATTTAGAATTGCT-3′ 5′-TGCCTTAAAC TTCCTTGCGAT-3′		[28]
13	5′ -CGA TCC CCT ATC AGC C-3′ 5′ -TCC TTA GAT AGA TGG TGT TGG-3′	5′ -TTC TGG TGT TGA CAA CAT GAC TAC TG-3′	[29]
14	5′ -GCG GTG TTT CGG TGA TTC ATA-3′ 5′ -TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-3′	5′ -AAA TTA GCG AAT CGC ATG GCT T-3′	[29]
15	TCG ACT TCC AGT TGG AGT CC AGC TCG ACC CAC TTA TCA C ATT GAC CAC GTC ACC ATC GA GTC CTT CTT GAG AAC GAG GT		[30]
16	5′ – CCG TGC TAA TTG TAG GGC TAA TAC A-3′ 5′ -GCT TGA AAC ACT CTA RTT TTC TCA AAG -3′		[31]
17	5′ CCA TACAACCCACTAGAG 3′, 5′ CTGTCATTTGGGTTTGATAG 3′, 5′ GACAACAGAGAGGTGATT 3′, 5′ CGTTGAATGTA ATGGGAAC 3′		[32]

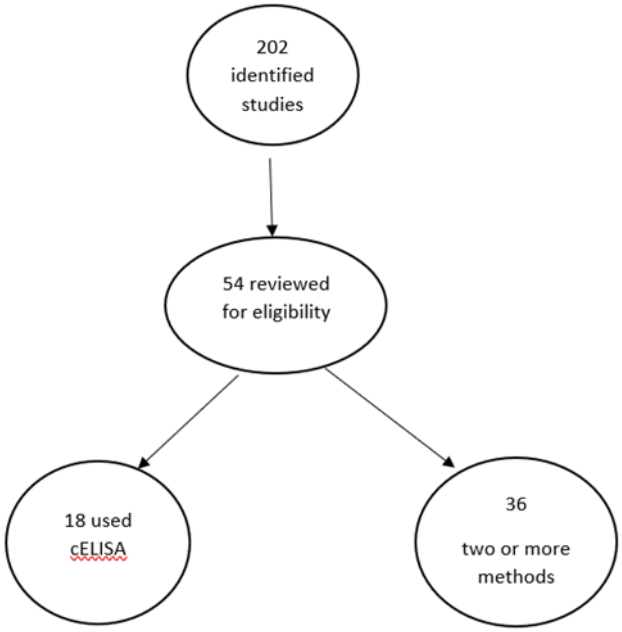

Figure 2. Selection process of studies using serological diagnostic methods.

Of the 54 articles identified based on serological diagnostic methods, 18 used cELISA as the diagnostic method and 36 achieved the aim of the study by at least two diagnostic methods, one of which was cELISA.

Identification of parasite species by direct microscopy was identified as one of the diagnostic methods used in 7 articles. In one study it was used as the only method, while in 6 articles it was used together with ELISA or PCR.

4. Discussion

T. equi, one of the main pathogens causing EPhas previously been subclassified into a number of clades based on sequence diversity of the 18S SSU rRNA gene. Current methods for clade-level genotyping of *T. equi* are laborious, PCR products must be generated, purified and sent for Sanger sequencing, and the presence of multiple allelic types in samples requires an additional molecular cloning step [22]. The 18S rRNA gene is a widespread target because nucleotide substitution rates are low and there is no evidence of lateral gene transfer between genetic lines [31]. Despite these facts, it can be observed that variable regions of this gene are often used for phylogenetic studies, in particular the 18S rRNA gene of *T. equi*, which has shown a high degree of sequence heterogeneity in different regions of the world [34].

After a primary infection with *T. equi* animals remain infected and become asymptomatic carriers with fluctuating levels of parasitaemia, a lifelong stage. Because parasitaemia levels fluctuate throughout the lifetime of the animal, the sensitivity of the duplex qPCR assay could be further improved by serial testing of initial cELISA positive/ qPCR negative tests [25].

In Romania, the first study using PCR on EP prevalence was conducted by Gallusová et al. in 2010-2012, which resulted in a prevalence of 25.4% for both piroplasma species from 18 localities inside and outside the Danube Delta [35].

It is important to note that different genotypes of *T. equi* (referred to as A-E) circulate in Europe, which may ultimately explain some differences in prevalence between countries, even though no link between genotype and virulence has been established so far [36].

In a study by Ribeiro et al. (2013), a 52% prevalence for *T. equi* infection was detected following PCR examination of 25 blood samples collected by jugular vein puncture and splenic puncture, respectively. The results of the study showed that 20% of the animals examined were positive in splenic puncture but negative in venous blood, while 28% were positive in jugular vein blood but negative in splenic puncture. Asymptomatic horses did not show parasitaemia but had infected erythrocytes in the spleen [37].

Development and validation of a new qPCR diagnostic protocol targeting the EMA-1 gene for *T. equi* and18S RNA for *B. caballi* was performed by Lobanov et al. (2018) demonstrating 100% specificity. In comparison, the samples under study were examined by both duplex qPCR and ELISA. Different results were

obtained for *B. caballi* by the two methods respectively 7.9% by qPCR and 58.6% by ELISA, which can be explained by the fact that *B. caballi* is eliminated after a period of time [25].

A study by Vieira et al. (2017) showed that 13.33% of seronegative tested animals were positive by PCR and 7.8% with negative PCR result were positive by ELISA. In this study, 7 horses were positive for *T. equi* by ELISA and negative for *T. equi* by nPCR. These are likely to be chronically infected carrier animals in which parasitaemia is below the detection threshold of molecular diagnostic techniques. A low and long-lasting parasitaemia could stimulate the immune system in animals that maintain serum antibodies at detectable levels [26].

Camino et al. (2019) performed a comparison between results obtained by several diagnostic methods namely cELISA, Real Time PCR, microscopic examination and haematological and biochemical screening. The study was carried out on 140 equines with specific clinical signs of EP and reported a prevalence of 9% by microscopic examination, while by cELISA and PCR the prevalence was 50.7% and 42.9% respectively [38].

Another study conducted in Iran by Abedi et al. (2019) on 106 apparently healthy horses resulted in a prevalence of 3.77% by direct microscopy and 50.94% by PCR.

Salinas-Estrella et al. (2022) compared in a study the results obtained by nPCR and duplex qPCR concluding that there was a relatively low concordance between nPCR and duplex qPCR for both piroplasma species and that it is also important to repeat the tests in serologically positive and molecularly negative animals and vice versa [39].

Ybañez et al. (2018) used blood smear, immunochromatographic test (ICT) and PCR as diagnostic methods for 105 Philippine horses, resulting in 23 animals positive for *T. equi* by ICT, 26 by PCR and no positive animals after examination of blood smears [24].

The cELISA technique is one of the most commonly used diagnostic methods in the diagnosis of EP being able to identify both carrier stage and acute infections, it is also simpler and less expensive than molecular techniques, but can still give false negative or false positive reactions, having a sensitivity of 95% and specificity of 99.5% [10].

Because seropositive animals in an asymptomatic population are not an indicator of recent or active infection, several authors have also tested seropositive samples by molecular methods to confirm or refute the presence of the piroplasm genome [40-46].

The use of direct microscopy as one of the diagnostic methods was identified in 7 of the articles reviewed. Positive results were presented in 4 studies [47,48,49] while for 3 articles the authors reported that no parasites were identified by this method [50,51,52].

The smear, from blood or lymph node, is a traditional method of agent identification in infected animals, but it is increasingly less used due to low specificity. The percentage of erythrocytes and leukocytes infected, in the clinical phase of the disease, with clinically expressed *T. equi* is between 1 and 5%, making identification on smear difficult [47].

Microscopic examination of smears is classified by the OIE as not suitable for testing the infection-free equine population and for use in contributing to eradication measures. This method is suitable in very limited circumstances for testing an individual infection-free animal, but is recommended with limitations for clinical confirmation of cases of EP [5].

5. Conclusions

Identification of the parasitic agent or infection can be done by direct methods, blood or lymph node smears during the acute phase of the disease, and by molecular and serological methods when in carrier animals the low parasite load makes detection extremely difficult.

Although some diagnostic methods may lack sensitivity and specificity, they are still widely used and useful to support clinical and epidemiological research.

Of all available serological methods, ELISA is the technique with the highest sensitivity and specificity, suitable for studying the prevalence of *T. equi* infections in equine populations. Serological methods are more sensitive compared to other diagnostic methods (clinical examination and direct microscopy) used, but even these techniques have limitations, e.g. they are not able to differentiate between current and previous infections.

Molecular diagnostic methods can overcome many of the limitations of other techniques and are essential to identify and distinguish genotypes of *T. equi*.

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References

1. Knowles, D.P.; Kappmeyer, L.S.; Haney, D.; Herndon, D.R.; Fry, L.M.; Munro, J.B.; Sears, K.; Ueti, M.V.; Wise, L.N; Silva, M.; Schneider, D.A.; Grause, J.; White, S.N.; Tretina, K.; Bishop, R.P.; Odongo, D.O.; Pelzel-McCluskey, A.M.; Scoles, G.A.; Mealey, R.H.; Silva, J.C. Discovery of a novel species, Theileria haneyi n. sp., infective to equids, highlights exceptional genomic diversity within the genus Theileria: implications for apicomplexan parasite surveillance. Int J Parasitol., 2018, 48(9-10):679-690.
2. Elsawy, B.S.M.; Nassar, A.M.; Alzan, H.F.; Bhoora, R.V.; Ozubek, S.; Mahmoud, M.S.; Kandil, O.M.; Mahdy, O.A. Rapid detection of equine piroplasms using multiplex PCR and first genetic characterization of Theileria haneyi in Egypt. Pathogens 2021, 10, 1414.
3. Phipps, L.P.; Otter, A. Transplacental transmission of Theileria equi in two foals born and reared in the United Kingdom. Vet Rec., 2004, 154:406–8.
4. Torres, R.; Hurtado, C.; Pérez-Macchi, S.; Bittencourt, P.; Freschi, C.; de Mello, V.V.C.; Machado, R.Z.; André, M.R.; Müller, A. Occurrence and Genetic Diversity of Babesia caballi and Theileria equi in Chilean Thoroughbred Racing Horses. Pathogens, 2021, 10(6):714.
5. OIE, Terrestrial Manual, Chapter 3.6.8, Equine Piroplasmosis, Available online: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.06.08_EQUINE_PIROPLASMOSIS.pdf (accessed on 03.03.2022).
6. Wagner, G.; Cruz, D.; Holman, P.; Waghela, S.; Perrone, J.; Shompole, S.; Rurangirwa, F. Non-imunologic methods of diagnosis of babesiosis, Mem. Inst. Oswaldo Cruz, Rio de Janeiro, 1992, Vol. 87, Suppl III, 193-199
7. Oladosu, L. A.; Olufemi, B. E. Haematology of experimental babesiosis and ehrlichiosis in steroid immunosuppressed horses. Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine, 1992, Series B, 39(5), 345–352.
8. De Waal, D. T.; Van Heerden, J.; Van den Berg, S. S.; Stegmann, G. F.; Potgieter, F. T. Isolation of pure Babesia equi and Babesia caballi organisms in splenectomized horses from endemic areas in South Africa. The Onderstepoort journal of veterinary research, 1988, 55(1), 33–35.
9. Lewis, M. J.; Wagner, B.; Woof, J. M. The different effector function capabilities of the seven equine IgG subclasses have implications for vaccine strategies. Molecular immunology, 2008, 45(3), 818–827.
10. Alanazi, A. D.; Alyousif, M. S.; Hassieb, M. M. (2012). Seroprevalence Study on Theileria equi and Babesia caballi Antibodies in Horses From Central Province of Saudi Arabia. Journal of Parasitology, 98(5), 1015–1017
11. Deepak, S.; Moudgil, A.D.; Singla, L.D. Equine Piroplasmosis: Current status. Veterinaria, 2014; 2(1): 9-14.
12. https://vmrd.com/test-kits/detail/theileria-equi-antibody-test-kit-celisa/ (accessed on 23.02.2022).
13. Nardini, R.; Bartolomé Del Pino, L. E.; Cersini, A.; Manna, G.; Viola, M. R.; Antognetti, V.; Autorino, G. L.; Scicluna, M. T. Comparison of PCR-based methods for the detection of Babesia caballi and Theileria equi in field samples collected in Central Italy. Parasitology research, 2021, 120(6), 2157–2164.
14. McPherson, R.A.; Pincus, M. R. Henry's Clinical Diagnosis and Management by Laboratory Methods - Polymerase Chain Reaction and Other Nucleic Acid Amplification Technology, State University of New York Downstate Medical Center, Brooklyn, New York, 2022, (69) 1271-1281.
15. Chifiriuc, M. C.; Gheorghe, I.; Czobor, I.; Florea, D.A.; Mateescu, L.; Caplan, M.E.; Caplan, D. M; Lazar, V. Advances in molecular biology based assays for the rapid detection of food microbial contaminants, , Food Preservation, 2017.
16. Ferreira, E.P.; Vidotto, O.; Almeida, J.C.; Ribeiro, L.P.S.; Borges, M.V.; Pequeno, W.H.C.; Stipp, D.T.; de Oliveira, C.J.B.; Biondo, A.W.; Vieira, T.S.W.J.; et al. Serological and molecular detection of Theileria equi in sport horses of northeastern Brazil. Comp. Immunol. Microbiol. Infect. Dis. 2016, 47, 72–76
17. Butler, C. Can Theileria equi be eliminated from carrier horses? Vet. J. 2013, 196, 279.
18. Torres, R.; Hurtado, C.; Pérez-Macchi, S.; Bittencourt, P.; Freschi, C.; de Mello, V.V.C.; Machado, R.Z.; André, M.R.; Müller, A. Occurrence and Genetic Diversity of Babesia caballi and Theileria equi in Chilean Thoroughbred Racing Horses. Pathogens, 2021, 10(6):714.
19. Zhao, S.; Wang, H.; Zhang, S.; Xie, S.; Li, H.; Zhang, X.; Jia, L. First report of genetic diversity and risk factor analysis of equine piroplasm infection in equids in Jilin, China. Parasit Vectors. 2020, 13(1):459.
20. Onyiche, T.E.; Taioe, M.O.; Ogo, N.; Sivakumar, T.; Biu, A.A.; Mbaya, A.W.; Xuan, X.; Yokoyama, N.; Thekisoe, O. Molecular evidence of Babesia caballi and Theileria equi in equines and ticks in Nigeria: prevalence and risk factors analysis. Parasitology, 2020,147(11):1238-1248.
21. Sunday Idoko I.; Tirosh-Levy S.; Leszkowicz M. M.; Mohammed A. B.; Sikiti G. B.; Wesley Nafarnda D.; Steinman A. Genetic Characterization of Piroplasms in Donkeys and Horses from Nigeria. Animals (Basel). 2020 Feb 18;10(2):324

22. Coultous, R.M.; McDonald, M.; Raftery, A.G.; Shiels, B.R.; Sutton, D.G.M.; Weir, W. Analysis of Theileria equi diversity in The Gambia using a novel genotyping method. Transbound Emerg Dis. 2020, 67(3):1213-1221.
23. Chisu, V.; Alberti, A.; Zobba, R.; Foxi, C.; Masala, G. Molecular characterization and phylogenetic analysis of Babesia and Theileria spp. in ticks from domestic and wild hosts in Sardinia. Acta Trop. 2019, 196:60-65.
24. Ybañez, A.P.; Ybañez, R.H.D.; Talle, M.G.; Arreglo, R.M.T.; Geens, M.J.C.; Villas, J.G.I. 3rd.; Villar, S.R.; Laruga, C.L.; Cao, S.; Moumouni, F.P.A.; Liu, M.; Igarashi, I.; Xuan, X. Serological and molecular detection of Theileria equi and Babesia caballi in Philippine horses. Ticks Tick Borne Dis. 2018, 9(5):1125-1128.
25. Lobanov, V.A.; Peckle, M.; Massard, C.L.; Brad Scandrett, W.; Gajadhar, A.A. Development and validation of a duplex real-time PCR assay for the diagnosis of equine piroplasmosis. Parasit Vectors. 2018, 11(1):125.
26. Vieira, M.; Costa, M. M.; de Oliveira, M. T.; Gonçalves, L. R.; André, M. R.; Machado, R. Z. Serological detection and molecular characterization of piroplasmids in equids in Brazil. Acta tropica, 2018, 179, 81–87.
27. Sumbria, D.; Singla, L.D.; Sharma, A.; Bal, M.S.; Randhawa, C.S. Molecular survey in relation to risk factors and haemato-biochemical alteration in Theileria equi infection of equines in Punjab Province, India. Vet Parasitol Reg Stud Reports. 2017, 8:43-50.
28. Malekifard, F.; Tavassoli, M.; Yakhchali, M.; Darvishzadeh, R. Detection of Theileria equi and Babesia caballi using microscopic and molecular methods in horses in suburb of Urmia, Iran. Vet Res Forum. 2014, 5(2):129-33.
29. Alanazi, A.D.; Said, A.E.; Morin-Adeline, V.; Alyousif, M.S.; Slapeta, J. Quantitative PCR detection of Theileria equi using laboratory workflows to detect asymptomatic persistently infected horses. Vet Parasitol. 2014, 206(3-4):138-45.
30. Sebastian, P.S.; Benitez-Ibalo, A.P.; Flores, F.S.; Debárbora, V.N.; Martinez, E.I.; Thompson, C.S.; Mangold, A.J. Molecular detection and phylogenetic characterization of Theileria equi in horses (Equus caballus) from a peri-urban area of Argentina. Ticks Tick Borne Dis. 2021, 12(6):101810.
31. Romero-Salas, D.; Solis-Cortés. M.; Zazueta-Islas, H.M.; Flores-Vásquez, F.; Cruz-Romero, A.; Aguilar-Domínguez, M.; Salguero-Romero, J.L.; de León, A.P.; Fernández-Figueroa, E.A.; Lammoglia-Villagómez, M.Á.; Becker, I.; Sánchez-Montes, S. Molecular detection of Theileria equi in horses from Veracruz, Mexico. Ticks Tick Borne Dis. 2021, 12(3):101671.
32. Sears, K.P.; Kappmeyer, L.S.; Wise, L.N.; Silva, M.; Ueti, M.W.; White, S.; Reif, K.E.; Knowles, D.P. Infection dynamics of Theileria equi and Theileria haneyi, a newly discovered apicomplexan of the horse. Vet Parasitol. 2019, 271:68-75.
33. Allsopp, M.T.E.P.; Allsopp, B.A. Molecular sequence evidence for the reclassification of some Babesia species. Ann. N.Y. Acad. Sci. 2006, 1081, 509–517.
34. Peckle, M.; Pires, M.S.; Silva, C.B.D.; Costa, R.L.D.; Vitari, G.L.V.; Senra, M.V.X.; Dias, R.J.P.; Santos, H.A.; Massard, C.L. Molecular characterization of Theileria equi in horses from the state of Rio de Janeiro, Brazil. Ticks Tick Borne Dis. 2018, 9(2):349-353.
35. Gallusova, M.; Qablan, M. A.; D'Amico, G.; Obornik, M.; Petrzelkova, K. J.; Mihalca, A. D.; Modry, D. Piroplasms in feral and domestic equines in rural areas of the Danube Delta, Romania, with survey of dogs as a possible reservoir. Veterinary Parasitology, 2014, 206(3-4), 287-292.
36. Tirosh-Levy, S.; Gottlieb, Y.; Fry, L.M.; Knowles, D.P.; Steinman, A., Twenty Years of Equine Piroplasmosis Research: Global Distribution, Molecular Diagnosis, and Phylogeny. Pathogens 2020,9, 926.
37. Ribeiro, I.B.; Câmara, A.C.; Bittencourt, M.V.; Marçola, T.G.; Paludo, G.R.; Soto-Blanco, B. Detection of Theileria equi in spleen and blood of asymptomatic piroplasm carrier horses. Acta Parasitol. 2013, 58(2):218-22.
38. Camino, E.; Cruz-Lopez, F.; de Juan, L.; Dominguez, L.; Shiels, B.; Coultous, R.M. Phylogenetic analysis and geographical distribution of Theileria equi and Babesia caballi sequences from horses residing in Spain. Ticks Tick Borne Dis. 2020, 11(6):101521.
39. Salinas-Estrella, E.; Ueti, M. W.; Lobanov, V. A.; Castillo-Payró, E.; Lizcano-Mata, A.; Badilla, C.; Martínez-Ibáñez, F.; Mosqueda, J. (Serological and molecular detection of Babesia caballi and Theileria equi in Mexico: A prospective study. PloS one, 2022, 17(3), e0264998.
40. Bělková, T.; Bártová, E.; Řičařová, D.; Jahn, P.; Jandová, V.; Modrý, D.; Hrazdilová, K.; Sedlák, K. Theileria equi and Babesia caballi in horses in the Czech Republic. Acta Trop. 2021, 221:105993.
41. Coultous, R.M.; Leadon, D.P.; Shiels, B.R.; Sutton, D.; Weir W. Investigating the presence of equine piroplasmosis in Ireland. Veterinary Record. 2020, 187(11): e97.
42. Bartolomé Del Pino, L.E.; Nardini, R.; Veneziano, V.; Iacoponi, F.; Cersini, A.; Autorino, G.L.; Buono, F.; Scicluna, M. Babesia caballi and Theileria equi infections in horses in Central-Southern Italy: Sero-molecular survey and associated risk factors. Ticks Tick Borne Dis. 2016, 7(3):462-9.
43. Laus, F.; Spaterna, A.; Faillace, V.; Veronesi, F.; Ravagnan, S.; Beribé, F.; Cerquetella, M.; Meligrana, M.; Tesei, B. Clinical investigation on Theileria equi and Babesia caballi infections in Italian donkeys. BMC Veterinary Research, 2015, 11:100
44. Moretti, A.; Mangili, V.; Salvatori, R.; Maresca, C.; Scoccia, E.; Torina, A.; Moretta, I.; Gabrielli, S.; Tampieri, M.P.' Pietrobelli, M., Prevalence and diagnosis of Babesia and Theileria infections in horses in Italy: a preliminary study. Veterinary Journal. 2010, 184(3):346-50.
45. Grandi, G.; Molinari, G.; Tittarelli, M.; Sassera, D.; Kramer, L.H. Prevalence of Theileria equi and Babesia caballi infection in horses from northern Italy, Vector Borne Zoonotic Dis. 2011, 11(7):955-6.
46. Butler, C. M.; Sloet van Oldruitenborgh-Oosterbaan, M. M.; Stout, T. A.; van der Kolk, J. H.; Wollenberg, L. V.; Nielen, M.; Jongejan, F.; Werners, A. H.; Houwers, D. J. Prevalence of the causative agents of equine piroplasmosis in the South West

of The Netherlands and the identification of two autochthonous clinical Theileria equi infections. Veterinary journal, 2012, 193(2), 381–385.
47. Padalino, B.; Rosanowski, S. M.; Di Bella, C.; Lacinio, R.; Rubino, G. Piroplasmosis in Italian Standardbred Horses: 15 Years of Surveillance Data. Journal of equine veterinary science, 2019, 83, 102813.
48. Díaz-Sánchez, A. A.; Pires, M. S.; Estrada, C. Y.; Cañizares, E. V.; Del Castillo Domínguez, S. L.; Cabezas-Cruz, A.; Rivero, E. L.; da Fonseca, A. H.; Massard, C. L.; Corona-González, B. First molecular evidence of Babesia caballi and Theileria equi infections in horses in Cuba. Parasitology research, 2018, 117(10), 3109–3118.
49. Abedi, V.; Razmi, G.; Seifi, H.; Naghibi, A. Molecular detection of equine piroplasms in donkeys (Equus asinus) in North Khorasan province, Iran. Iranian journal of veterinary research, 2015, 16(2), 202–204.
50. Campos, J.; André, M. R.; Gonçalves, L. R.; Freschi, C. R.; Santos, F. M.; de Oliveira, C. E.; Piranda, E. M.; de Andrade, G. B.; Macedo, G. C.; Machado, R. Z.; Herrera, H. M. Assessment of equine piroplasmids in the Nhecolândia sub-region of Brazilian Pantanal wetland using serological, parasitological, molecular, and hematological approaches. Ticks and tick-borne diseases, 2019, 10(3), 714–721.
51. Nugraha, A. B.; Cahyaningsih, U.; Amrozi, A.; Ridwan, Y.; Agungpriyono, S.; Taher, D. M.; Guswanto, A.; Gantuya, S.; Tayebwa, D. S.; Tuvshintulga, B.; Sivakumar, T.; Yokoyama, N.; Igarashi, I. Serological and molecular prevalence of equine piroplasmosis in Western Java, Indonesia. Veterinary parasitology, regional studies and reports, 2018, 14, 1–6.
52. Souza, E.; Araujo, A. C.; Pires, L.; Freschi, C. R.; Azevedo, S. S.; Machado, R. Z.; Horta, M. C. Serological detection and risk factors for equine piroplasmosis in the semiarid region of Pernambuco, Northeastern Brazil, Brazilian journal of veterinary parasitology, 2019, 28(4), 685–691.