Journal of Animal Science/vol.30, No.4; 2021/pp 95-101

Detection of *Theileria annulata* infected carrier cattle by molecular assay in northwest of Iran

A Nematollahi[1*], P Shahbazi[2], S Ebrahimian[3] and B Hassanzadeh[4]

Received: November 18, 2020 Accepted: January 9, 2021

[1]Professor, Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[2]Associated professor, Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[3]MSc, Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[4]Assistant professor, Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
[*]Corresponding author E-mail: anemat@tabrizu.ac.ir

Journal of Animal Science/vol.30 No.4/ 2021/pp 95-101
https://animalscience.tabrizu.ac.ir

DOI: 10.22034/as.2021.42894.1594

Abstract

Bovine theileriosis, caused by *Theileria annulata*, is one of the most important livestock diseases in the world. The disease is transmitted by Ixodidae ticks and characterized by fever, enlargement of lymphatic glands, cachexia, and death. theileriosis can be detected by staining the blood smear Giemsa dye. The technique is suited the detection of acute infection but not in carrier animals, where infection rate may be little. The aim of present study was to detect *T. annulata* carrier cattle by polymerase chain reaction (PCR) and to compare the results with staining method in the northwest of Iran. **Materials and Methods:** In this study, peripheral blood samples were obtained from 281 apparent healthy cattle (no clinical signs of disease) of different ages (1 to 8 years old). Samples were tested by two techniques of (1) Giemsa staining and microscopy and (2) PCR based on applying the specific primers from the major merozoite-piroplasm surface antigen sequence of *T. annulata* (Tams-1) gene. A chi-square test was performed to compare the prevalence related to breed and age categories. **Results:** In this study, 25 *T. annulata* positive samples (8.89%) were detected by microscopic method while PCR was able to detect 108 samples (38.43%) for. In positive samples of cattle, the highest prevalence was recorded for 2-5 years old cattle (22.4%). These differences in age results were significant ($P<0.05$). Out of 108 positive PCR samples, 45 (41.66%) were native and, 63 (58.33%) were crossbreed cattle, yet the difference was statistically insignificant. **Conclusion:** Our results showed that there is a high percentage of carrier cows in northwest of Iran and indicate a high potential risk for the infestation of healthy animals and vectors of the disease.

Keywords: Carrier, Iran, Staining method, PCR, *Theileria annulata*

Introduction

Tropical theileriosis caused by *Theileria annulata* is one of the most devastating blood parasites affecting cattle, sheep and other livestock animals. The disease is characterized by fever, loss of weight, lymphadenopathy,

anemia, and death. *T. annulata* is transmitted by Ixodid tick's genus *Hyalomma* (Khattak *et al.* 2012). Life cycle of the protozoa is carried in both vertebrate and invertebrate hosts. Sporozoites of *Theileria* enter bovine through tick infestation and attack mononuclear leukocytes, develop macroschizonts and enforce host cell proliferation. Macroschizonts are transferred to microschizonts and subsequently to merozoites invading erythrocytes and developing into "Piroplasmic forms" in the cattle blood. The above-mentioned clinical features result from pirosplasmic forms (d'Oliveira *et al*. 1995; Khattak *et al*. 2012; Shahnavaz *et al*. 2011) Generally, detection of theileriosis in cattle is based on identification of piroplasmic forms of parasite in the blood smears and macroschizonts in the biopsy smears of enlarged lymph nodes by Giemsa staining by. The technique is suited diagnosing acute infection but are not in carrier animals, where infection rate may be little (Altay *et al*. 2008; Minjauw *et al*. 2003). Moreover, serological tests such as the direct immune-florescent antibody test (IFAT) can be used for detecting the secretory antibodies of the disease. Nonetheless, its practice is limited due to (1) cross-reactivity with antibodies directed against other species of *Theileria* spp., and (2) disappearance of antibodies in chronic carriers (Dumanli *et al*.2005; Schnittger *et al*.2004). Clinical findings and microscopic examination of the stained blood smears in acute cases of theileriosis are adequate. However, the technique becomes inadequate where low infection rate leads to a carrier state whether in native and or treated cattle. Such carriers react as the transferor of infestation throughout the ticks. Biligic *et al*. (2013) believed that "when the carrier cattle transfer to non-endemic regions, this can lead to the onset of theileriosis" (Bilgic *et al*.2013). Species-specific molecular diagnostic procedures such as Polymerase Chain Reaction (PCR) due to high sensitivity and specificity are considered superior to the microscopic diagnostic method, (Thillampala *et al*.2012). The objective of this investigation was the detection of *T. annulata* carrier cattle by PCR and its comparison to the staining method in the northwest of Iran.

Materials and methods

Sample collection

The 281 blood samples from ear (peripheral) veins were taken from 101 native cows and 180 crossbred cattle in the North West of Iran from July to September, 2019. The age range of cows was 1-8 years. Samples were obtained from animals showing no clinical signs. Blood samples were kept in micro tubes containing a few drops of 0.5 M EDTA.

Giemsa staining

Methanol fixed blood smears were prepared and stained using Giemsa (Merck, Germany), and studied by a light microscope (Olympus, Japan). The piroplasmic form of *T. annulata* was diagnosed according to a method described by Soulsby (Soulsby 1985). The piroplasmic form of *T. annulata* was distinguished as a ring shaped in red blood cells,2µm by 0.5-1 µm.

Blood DNA Isolation

DNA was isolated by the method of Ghatak et al. (2013) and Bartlett (2003) with a few modifications (Ghattak et al., 2013; Bartlett et al., 2003). Ammonium bicarbonate and ammonium chloride were used for blood cells; lyses, and lymphocytes were harvested by the destruction of the red blood cells (RBC). Briefly, RBC were lysed using RBC lysis buffer by thorough shaking and reversing for 5 min and then centrifugation at 2000 g for 10 min. The supernatant was discarded, leaving 1 ml to avoid the loss of lymphocytes. Lymphocytes were washed two more times with 3 µL of RBC lysis buffer was added, until a clear supernatant. For DNA extraction, the pellet was re-suspended in 500 µl PBS then by adding 400 µl of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5) and 15 µl proteinase K (10 mg/ml stock; Bioneer, S. Korea) lymphocytes were lysed. The samples were shaken vigorously to liquefy the cell plaque completely and then placed in56˚C water bath for two hours. The aqueous phase of samples was separated by using the same volume (1:1) of phenol (equilibrated with Tris, pH 8) and chloroform: isoamyl alcohol

(24:1) and lysed cells. Tubes were centrifuged at 10,000 g (at 4˚C) for 10 min, and then the aqueous phase was transferred into a new micro tube. DNA was precipitated by applying µL of absolute alcohol (Merck) to the tubes and keeping them in -20˚C, and then, tubes were centrifuged at 10,000 g (4˚C) for 10 min. The upper phase was discarded, by adding 250 µl of 70% ethanol, and the pellet was dislodged smoothly by pipetting. Tubes spun at 10,000 rpm for 10 min. The resulting DNA plaque was air-dried on a dry bath, and subsequently reconstituted in 50 µl TE buffer, and stored at -80˚C for further analysis. The purity and integrity of extracted DNA were assessed with submerged gel electrophoresis.

Processing of Polymerase chain reaction

A pair of published primer was used to amplify a segment of merozoite surface antigen gene (Tams 1) from *T.annulata* as described by d'Oliviera et al. (1995) [1].The primer-pair nucleotide sequences were (N516) 5'GTAACCTTTAAAAACGT3' and (N517) 5'GTTACGAACATGGGTTT3'. The PCR reaction was performed in a final volume of 25 µl containing 12.5 µl Master mix (Biotech), 1 µl extracted DNA sample,1.5 µl of each primer including forward and reverse (10pmol/µl) and, 8.5 µl nuclease-free water. Sterile water as negative control and positive control were used in the amplification run. PCR was carried out in a thermal cycler (Biotech, South Korea) according to the aforementioned schedule. PCR reactions were initialed at 95 ˚C for 5 min, followed by 94 ˚C for 45 s, 58 ˚C for 1min, 72 ˚C for 1min, and finalized at 72 ˚C for 10 min.

Detection of PCR products in gel electrophoresis

Products of PCR were examined by agarose gel electrophoresis on 1.5% gel and then stained by Safestain®(Merck, Germany) and finally documented by a gel documentation system (Bioluminators).

Statistical analysis

Man-Whitney U test was used for assessing the association between microscopy and PCR. Chi-square test was performed to compare the prevalence of diseases related to breed and age categories. Differences were considered statistically significant when $P<0.05$. Data was analyzed using SPSS (Version 25).

Results

Blood smear examination

Out of Giemsa stained 281 blood smears, 25 (8.89%) were *T.annulata* positive. In the microscopic examination of Giemsa stained blood smears, Piroplasmic forms were observed as ring form and 2µm by 0.5-1 µm (Fig1).

Polymerase chain reaction

PCR results indicated that 108 (38.43%) from 281 tested blood samples produced 721bp fragment specific for *T.annulata*. All positive samples in Giemsa staining were positive in PCR too. The PCR was based on the specific primers from the merozoite surface antigen sequence of the *T.annulata* (Tams 1) gene (Fig. 2).

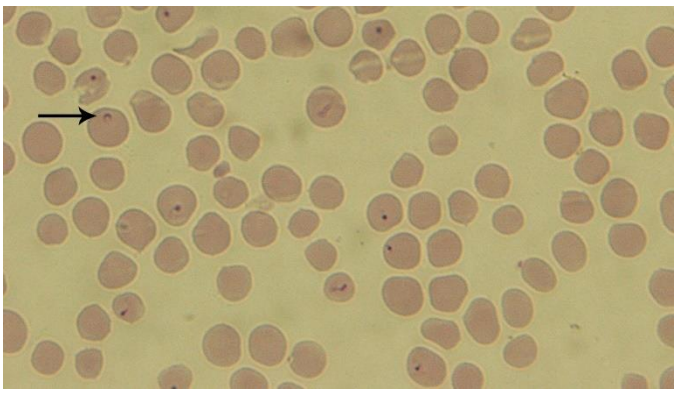

Figure1- Piroplasmic forms of T. annulata in microscopic examination by Giemsa stained blood smears (×100)

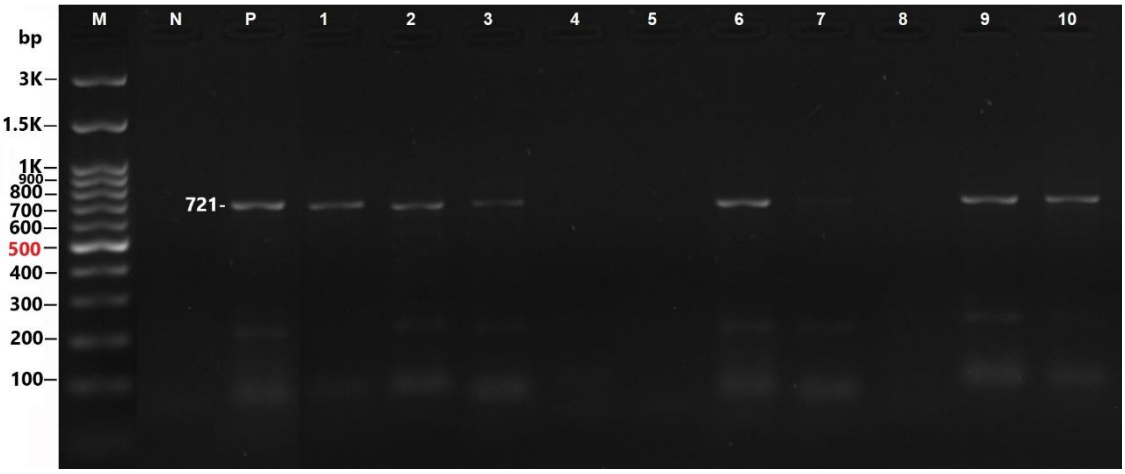

Figure 2-PCR product run on 1.5% agarose gel, M; marker, N: Negative control, P: Positive control,1,2,3,6,7,9,10: Positive samples,4,5,8: Negative samples.

In positive samples of cattle, the maximum prevalence was obtained in 2 to 5 years old cattle (22.4%) (Table 1). The differences in age results were significant. Out of PCR detected positive samples (108 samples), 45 (41.66%) were native, and 63 (58.33%) crossbreed cattle but the difference was insignificant. In statistical analysis observed value of the Man-Whitney U test was 0.001, and it is sufficient ground for accepting that PCR is more efficient in blood samples suspected to theileriosis compared to the microscopic examination of Giemsa stained samples.

Table 1-The age distribution in positive cattle by PCR

Age	Number of cows tested	Positive by PCR	Total Positive (%)
<1 year	24	10	3.55
1-2 year	105	24	8.54
2-5 year	117	63	22.4
>5year	35	11	3.91
Total	281	108	38.43

Discussion

Theileriosis due to *T.annulata* is more important between seven species of *Theileria* spp. in tropical zones of the world. This disease is characterized by clinical symptoms such as anorexia, decrease rumination, nasal discharges, lymphadenopathy, and death. Detection of theileriosis is achieved by clinical signs and examination of blood smears stained with Giemsa by microscope. The sensitivity of this method is adequate, but accuracy may lead to false diagnosis. Also, in acute infections, in which piroplasmic forms of the parasite are not formed yet and, in the detection of carrier animals, this method was adequate. Next, investigations proposed serological tests such as Immunofluorescent Assay Test (IFAT) and Indirect Haemagglutination Assay (IHA), and Enzyme likaged Immunosorbent Assay (ELISA). However, recent studies have shown that serological tests lack sensitivity in theileriosis endemic regions when tick transmission is seasonal (Kirvar *et al*. 2000; Kundave *et al*. 2013). Also, the method is not capable of detecting early but patent infections. Furthermore, cross-activity with antibodies directed produced by other *Theileria* spp. decrease the specificity of serological tests, especially IFAT. Moreover, in long-term carrier animals, antibodies disappear, whereas infection persists, and the animals can still infect ticks (Dumamli *et al*. 2005).

Several studies in recent decay documented

that molecular methods such as PCR are highly specific and sensitive than regular methods for diagnosing theileriosis and detection of carrier cattle, which have not any obvious signs of theileriosis (d'Oliveira *et al*. 1995).

In this study, we fixed a PCR method for the diagnosis of *T.annulata* in blood samples of carrier cattle. A 721bp product was amplified from blood samples from 108 out of 281 cattle by applying primer set N516/517. This study confirmed the earlier studies that molecular techniques are more sensitive than the microscopic survey in the diagnosis of theileriosis as only 28 out of 281 (8.89%) blood samples were detected to be infested to the parasite by staining method whereas, by PCR method 108(38.43%) samples were positive (Hoghooghi-rad *et al*.2011). All positive samples were also detected by the PCR amplification method. The infection rate of *T.annulata* which obtained by PCR in this study, indicate that infection in the northwest of Iran is moderate (38.43%), and this finding is similar to the finding of Aziz *et al*. (2007), who detected a 40% infection rate of 140 carrier cattle by PCR and only 8.1% by Giemsa staining method(Azizi *et al*.2007).But Hoghooghi-Rad *et al*. (2011) used a semi-nested PCR method to determining carrier cattle in Golestan (Iran) in 160 blood samples, and they reported this method revealed 12 (7.5%) positive; the staining method could diagnose 6(3.75) out of 160 blood samples (Hoghooghi-rad *et al*. 2011). This finding is similar to our finding of the sensitivity of molecular methods compared by the staining method, whereas it is not coordinate with the rate of infection in our study. This difference is probably due to different conditions the environment and the diversity of ticks as intermediate hosts of theileriosis. Similar research was done by Nourollahi-Fard *et al*. (2013) where they showed schizont forms of *T.annulata* in 16 of 150 (10.66%) by testing the blood smears, whereas 68 of 150 (45.33 %) cattle were infected by the PCR method in Kerman (Iran) (Nourollahi-Fard *et al*. 2015). The rate of theileriosis in the present study in 2 to 5 years old cattle is significantly higher than in other age groups. Also, the infection rate to theileriosis in this study in native cattle was more heightened than crossbreed. Similar studies indicate that the prevalence of theileriosis in native cattle is high and asymptomatic infection in local breeds can be infectious sources to more sensitive breeds. This phenomenon may be attributed to the high sensitivity of native cattle than crossbreed cattle and management of breeding. The native cattle are at risk of tick biting than a crossbreed and, the population of ticks in native breeding farms is more than industrial.

The present study demonstrated that many cattle in our area were positive but were asymptomatic carriers. This strongly suggests that native cattle in North West of Iran are resistant to theileriosis. Crossbred cattle from Holstein and native breeds also were asymptomatic. Therefore, the crossbred cattle raised in these areas may be genetically resistant from the local breeds. But, is discovered that "carrier" crossbreed cattle showed a higher level of haematological pathology when infected with *T. annulata*..(Larcombe *et al*.2019). In conclusion, based on our findings, PCR was rather more sensitive diagnosis tool than the microscopy since low rate of *T.annulata* could also be detected by this technique. Also, the carrier rate of theileriosis is high in the northwest of Iran, and it is a high potential risk for the infestation of healthy animals.

Conflict of interest

All authors have received research grants from University of Tabriz, Iran, and declare that they have not conflict of interest.

Funding information

This Research Project was fully sponsored by University of Tabriz, Iran.

References

Altay K, Aydin MF, Dumanli N and Aktas M, 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. Veterinary Parasitology 158(1-2) 295-301.

Azizi H, Shiran B, Farzaneh-Dehkordi A, Salehi F and Taghadosi C,2007. Detection of *Theileria annulata* by PCR and its comparison with smear method in native carrier cows. Biotechnology 7: 574-577.

Bartlett JMS and Striling D, 2003. Extraction of DNA from whole blood in: Methods in molecular biology, PCR protocols.2nd ed. Totowa,NJ: Humans Press Inc.

Bilgic BH, Karagenc T, Simuunza M, Shiels B, Tait A, Eren H and Weir W,2013. Development of multiplex PCR assay for simultaneous detection of *Theileria annulata, Babesia bovis* and *Anaplasma marginale* in cattle. Experimental Parasitology 133:(2) 222–229.

d'Oliveira C, Van-der Weide M, Habela A, Jacquiet P, Jongejan F,1995. Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. Journal of Clinical Microbiology 33(10):2665–2669.

Dumanli N, Aktas M, Cetinkaya B, Cakmak A, Koroglu E, Saki CE, Erdogmus Z, Nalbantoglu S, Ongor H, Simsek S, Karahan M,Altay K, 2005.Prevalence and distribution of tropical theileriosis in eastern Turkey. Veterinary Parasitology 127(1):9–15.

Ghatak S, Muthukumaran RB, Nachimuthu SK, 2013. A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. Journal of Biomolecular Techniques 24(4):224-225.

Hoghooghi-Rad N, Ghaemi P, Shayan P, Eckert B, Sadr-Shirazi N,2011. Detection of native carrier cattle infected with *Theileria annulata* by Semi-bested PCR and smear method in golestan province of Iran. World Applied Sciences Journal 12(3): 317-323.

Khattak R, Hameed H, Taqddus A, Faryal M, Durranis S, Gillani QUA, Allahyar R, Shaikh RS,2012. A comparison of two different techniques for the detection of blood parasite, *Theileria annulata*, in cattle from two districts in Khyber Pukhtoon Khwa Province (Pakistan). French Society of Parasitology 19(1): 91.

Kirvar E, Ilhan T, Katzer F, Hooshmand-rad P, Zweygarth E, Gerstenberg C, Phipps P, Brown CG,2000. Detection of *Theileria annulata* in cattle and vector ticks by PCR. Parasitology 120(3):245–254.

KundaveVR, Patel PV,Hasani JJ,Joshi CG,2013. Detection of theileriosis in cattle and buffaloes by Polymerase chain reaction. Journal of parasitic diseases 39(3): 508–513.

Larcombe SD, Kolte SW, Ponnudurai G, Kurkure N, Magar A, Alagesan A, Weir, Shiels BR, 2019. The impact of tick-borne pathogen infection in Indian bovines is determined by host type but not the genotype of *Theileria annulata*. Infection, Genetics and Evolution 75:103-109.

Minjauw B and Mc Leod A, 2003. Tick-borne diseases and poverty: the impact of ticks and tick-borne diseases on the livelihood of small scale and marginal livestock owners in India and eastern and southern Africa. Animal Health Program, DFID Animal Health Program:1–124

Nourollahi-Fard SR, Khalili M, Ghalekhani, N,2015. Detection of *Theileria annulata* in blood samples of native cattle by PCR and smear method in Southeast of Iran. Journal of Parasitic Diseases 39(2):249-52.

Shahnavaz S, Ali M, Aslam MA, Fatima R, Chaudhry ZI, Hassan MU, Ali M, Iqbal F,2011. A study on the prevalence of a tick transmitted pathogen, *Theileria annulata*, and hematological profile in cattle from Southern Punjab. Parasitology Researches 109(4):1155-1160.

Schnittger L, Yin H, Qi B, Gubbels M J, Beyer D, Niemann S, Jongejan F, Ahmed J S,2004. Simultaneous detection and differentiation of *Theileria* and *Babesia* parasites infecting small ruminants by reverse line blotting. Parasitology Researches 92(3):189–196.

Soulsby EJL, 1982. Helminthes, arthropods and protozoa of domesticated animals, 7th ed. London: Bailliere Tindall.

Thillampala S, Hamel K, Sembulutti AEA, Singarayer CV, Asela SM, Dilhani TH, Thamotharampillari O, Subramaniyam S, Kulanayagam K, Alawattage DNC, Ikuo I,Naoaki Y,2012. A PCR-based survey of selected *Babesia* and *Theieria* parasites in cattle in SriL anka. Veterinary Parasitology 190(1-2):263-267.

DOI: 10.22034/as.2021.42894.1594

تشخیص گاوهای حامل آلوده به تیلریا آنولاتا به روش مولکولی در شمال غرب ایران

احمد نعمت الهی*[1]، پریسا شهبازی[2]، صمد ابراهیمیان[3] و بلال حسن زاده[4]

تاریخ دریافت: ۹۹/۸/۲۸ تاریخ پذیرش: ۹۹/۱۰/۲۰

[1] استاد گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه تبریز
[2] دانشیار گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه تبریز
[3] کارشناس ارشد انگل شناسی، دانشکده دامپزشکی، دانشگاه تبریز
[4] استادیار گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه تبریز
*مسئول مکاتبه: Email: anemat@tabrizu.ac.ir

چکیده

زمینه مطالعاتی: تیلریوز گاوی ناشی از تیلریا آنولاتا یکی از بیماریهای مهم در حیوانات مزرعه در جهان می باشد. این بیماری توسط کنه های ایکسودی ده منتقل شده و با تب، بزرگی غدد لنفاوی، لاغری مفرط و مرگ مشخص می شود. تشخیص این بیماری بر اساس رنگ آمیزی گسترشهای خونی به روش گیمسا انجام می گیرد. این روش برای تشخیص عفونت حاد مناسب می باشد اما برای تشخیص حاملین بیماری که میزان آلودگی آنها کمتر می باشد مناسب نیست. **هدف**: این مطالعه جهت تشخیص حاملین بیماری ناشی از تیلریا آنولاتا توسط نمونه های خونی و مقایسه آن با روش مولکولی PCR در گاوان شمالغرب کشور انجام پذیرفت. روش کار: تعداد ۲۸۱ نمونه خونی از گاوان بومی و دو رگه به ظاهر سالم با سنین مختلف در شمالغرب کشور در تابستان سال ۱۳۹۸ اخذ شد. نمونه ها بعد از تهیه گسترش و رنگ آمیزی به روش گیمسا در زیر میکروسکپ نوری بررسی شدند و روش واکنش زنجیره ای پلیمراز (PCR) با استفاده از پرایمر های تهیه شده از آنتی ژن سطحی مروزوئیت انگل که ژن (Tams-1) را کد می کنند انجام شد. آزمون مربع کای جهت جهت مقایسه میزان شیوع بیماری در ارتباط با نژاد و سن حیوانات کار گرفته شد. **نتایج**: در روش میکروسکپیک تعداد ۲۵ نمونه (۸٪/۸۹) نمونه ها از لحاظ آلودگی انگلی مثبت تشخیص داده شد ولی در روش PCR ۱۰۸ نمونه (۳۸/۴۳٪) نمونه ها مثبت تشخیص داده شد. در نمونه های مثبت گاوان بیشترین میزان شیوع در گاوان ۵-۲ ساله (۲۲/۴ درصد) مشاهده شد که این تفاوت معنی دار بود($P<0.05$). در بین ۱۰۸ نمونه مثبت تشخیص داده شده با PCR ۴۵ مورد (۴۱/۶۶ درصد) در گاوان بومی و ۶۳ مورد (۵۸/۳۳ ٪) در گاوان دورگه شناخته شد. **نتیجه گیری**: نتایج بررسی حاضر میزان بالای گاوان حامل بیماری تیلریوزیس را نشان داد که این موضوع نشانگر پتانسیل بالای خطر آلودگی برای حیوانات سالم و ناقلین بیماری می باشد.

واژگان کلیدی: ایران، تیلریا آنولاتا، حامل، روش رنگ آمیزی، PCR