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# Molecular Characterisation of *Anaplasma marginale* and *Theileria orientalis* in Slaughtered Bovines of Aizawl District, Mizoram

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## ABSTRACT

**Background:** Bovines are domesticated ungulates mainly raised as livestock for milk, meat and leather and as draught animals. A slaughterhouse study helps to assess the disease status of herds and contains valuable information about the incidence and epidemiology of animal diseases. Haemoprotozoan diseases are one of the major problems which adversely affect the health and productivity of cattle. The diverse climatic zones of India are highly conducive to the survival and propagation of vectors and vector-borne pathogens. However, there is no data on proper slaughterhouse study of bovines in Mizoram even in India for determination of the prevalence of haemoprotozoan infections.

**Methods:** The blood samples were collected in an EDTA vials. The blood smear was prepared, stained with Giemsa stain and examined for the presence of haemoprotozoan. The DNA was isolated from positive samples by use of a DNeasy Blood and Tissue Kit. Then PCR was performed for all the isolated DNA collected from positive samples. After confirmation through PCR, the representative samples for *Anaplasma sp.* and *Theileria sp.* were sent for sequencing. Then the sequencing results were annotated using BLAST, ClustalW multiple alignment program of MEGA9 software. The phylogenetic tree was generated by using the Neighbour-Joining method, keeping bootstrap consensus from 1000 replicates.

**Result:** After the blood smear examination, PCR was performed for confirmation. Molecular detection of *Anaplasma marginale*, *Theileria orientalis* was done. Molecular characterization of 2 isolates from *Anaplasma marginale* and *Theileria orientalis* was performed by sequencing. The sequence analysis of *rpoB* gene of isolates of *Anaplasma* revealed both the isolates were placed in the same clade in the phylogenetic tree. The phylogenetic analysis of *Thileria* isolates revealed that isolate Miz-86 was closely placed with the Type-N3 clade. Whereas the other isolate *i.e.* Miz-87, belonged to the Type-5 clade.

**Key words:** Aizawl, Bovines, Mizoram, Pathology, Slaughterhouse.

## INTRODUCTION

Cattle and buffaloes are domesticated ungulates, members of the subfamily *Bovinae* and family *Bovidae*. The animal husbandry department contributed a major part to the Indian economy and overall contribution is 28-32% of agricultural GDP (Gross Domestic Product) and 4-6% of the national GDP. India possesses the largest livestock population in the world with 536 million of domesticated animals; the total bovine population is 302.79 million (Anonymous, 2019). The rearing of bovines in North Eastern has great importance related to the economy but a lot of limiting factors come into play in the way of ensuring profitability and sustainable productivity from these animals. The effective development of any livestock industry depends upon the prevention and control of diseases among animals (Singh *et al.,* 2000).

Parasitic diseases are one of the major problems which adversely affect the health and productivity of cattle (Bhatnagar *et al*., 2015). The exotic and cross breeds of animals reared in India are of more susceptible to tick infestation and thereby tick-borne diseases compared to indigenous animals (Kumar *et al*., 2015). The diverse climatic zones of India are highly conducive to the survival and propagation of vectors and vector-borne pathogens (Bhattacharjee and Sarmah, 2013; Laha *et al.,* 2015). The two most important haemoprotozoan diseases encountered in cattle are babesiosis and theileriosis (Rajput *et al*., 2005). Anaplasmosis, one of the most important rickettsial diseases

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occurred in cattle, has also a great impact on animal productivity (Radostitis *et al.,* 2000). Age plays most important role in endemicity of *Anaplasma marginale* infections in bovines (Isik *et al.,* 2018).

Abattoirs have an important role mainly in the surveillance of various diseases of animals and human beings (Alton *et al.,* 2010). Abattoir study also plays a significant role to know about the extent of exposure of the public to certain zoonotic diseases and helps in the estimation of the financial losses due to condemnation of affected organs (Cadmus and Adesokan, 2009; Raji *et al.,* 2010; Singla and Juyal, 2014). In India, slaughters of bulls and cow are allowed by the Government in states like Arunachala Pradesh, Assam, Goa, Kerala, Mizoram, Meghalaya, Nagaland, Tripura and West Bengal. The present study was conducted as there is no proper data regarding the incidence of haemoprotozoan infections in bovines that are slaughtered in Mizoram or any other part of India.

## MATERIALS AND METHODS

Slaughterhouse was regularly visited and blood samples were randomly collected in EDTA vials from slaughtered bovines during the period from January 2020 to September 2020 for the study of different hemoparasitic infections. The collected blood samples were properly packed in ice and brought to the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Central Agricultural University, Mizoram, India for further examination.

### Blood Smear examination

Thin smears were prepared on clean grease-free slides from the anticoagulated blood samples and were fixed with absolute methanol for 30 seconds. Fixed smears were stained with 10% Giemsa stain for 45 mins. Then the smear was examined for the presence of haemoprotozoan (Schalm *et al*., 1975).

### Genomic DNA isolation from blood samples

Blood samples positive for haemoparasitic infection in blood smear examination were used for DNA isolation which was used in PCR assay later. DNA extraction was carried out by use of DNeasy Blood and Tissue Kit (Qiagen®, Germany, Catalogue No. 69504) as per the manufacturer's protocol/ instructions and stored at -20°C for further use.

### PCR assay

The PCR reaction was carried out in 12.5 µl of 10X PCR green buffers (Thermo Scientific, USA) containing 0.1 µl of Taq DNA polymerase, 0.5 µl of each primer (10pmol/µl) and 0.3 µl of 10mM dNTPs and 1 µl of template DNA. Amplification was done in C1000 thermal cycler (Bio Rad, USA) and the amplicons were checked in agarose gel electrophoresis using 1.5% agarose gel. The gel was then visualized and documented in a Gel documentation system.

### Cloning, nucleotide sequencing and Phylogenetic analysis

Out of all positive blood samples for *Anaplasma marginale* and *Theileria orientalis* confirmed by PCR, two samples each from the above species were randomly selected and cloning was performed by using InsTAcloneTM PCR cloning kit (Thermo Scientific, USA). The purified PCR products were ligated into thepTZ57R/T Cloning vector (Thermo Scientific, USA). The recombinant plasmids were transformed into DH5α *E. coli* cells. The positive clones (containing the inserts) were confirmed by Colony PCR which were then stab inoculated in LB stab culture containing ampicillin and were incubated overnight and sent to the Department of Biochemistry, Mizoram University, Mizoram, for sequencing. Then the sequencing results were annotated using BLAST, ClustalW multiple alignment program of MEGA9 software. The phylogenetic tree was generated by using the Neighbour-Joining method, keeping bootstrap consensus from 1000 replicates. For comparison, sequence data retrieved from GenBank was considered for generating the phylogenetic tree. Subsequently, the annotated sequences were submitted to GenBank, NCBI to get an accession number.

## RESULTS AND DISCUSSION

A total of 80 no. of blood samples were collected during the study period from January 2020 to September 2020 from the slaughtered bovines. The overall incidence of hemoprotozoan infections was 33.75% (*i.e.* 27/80) which was nearly the same as the previous study (*i.e.* 33.30%) by Ghosh *et al*., 2018 in Mizoram. But Velusamy *et al*. (2014) recorded an overall prevalence of 16.64% of hemoparasitic infections in Tamil Nadu, India and in another study by Bhatnagar *et al*. (2015) in Rajasthan, India, the overall prevalence was 9%. The difference in the results may be due to variations in the climatic and geographical conditions of the study areas and the managemental practices of animals. Among these, *Anaplasma sp*. Infections showed the highest incidence *i.e*. 18.75% (15/80) followed by *Theileria sp*. Infections *i.e.* 15.0% (12/80). Kakati *et al.* (2015) reported that the prevalence of Anaplasmosis was 14.03% and of Theileriosis was 21.05% in Assam. Patra *et al*. (2021) revealed mixed infection of both *Anaplasma sp.* and *Theileria sp.* in their study. Maharana *et al*. (2016) also reported Anaplasma sp. infection in cattle and buffaloes of South-west Gujarat, India. *Anaplasma sp.* appeared as round, oval, or disc-like deep red-colored bodies either in the centre or in the margin of erythrocytes (Fig 1A). *Theileria sp.* were pleomorphic and appeared mostly round or annular inside erythrocytes (Fig 1B) whereas, in lymphocytes, they appeared as oval or comma shape (Behera *et al.,* 2022).

Blood samples examined microscopically for hemoprotozoan infections were further confirmed by molecular method. These samples were detected by PCR for the *rpoB* gene of *Anaplasma marginale*, a gene encoding

a polymorphic merozoite/piroplasms surface protein (MPSP) of *Theileria orientalis*. Fifteen samples were found positive for *Anaplasma marginale* whereas 12 samples were positive for *Theileria orientalis*. A 576 bp size of a fragment of the *rpoB* gene was amplified as per Dahmani *et al*., 2017 (Fig 2). A 776 bp size of a fragment of gene encoding a polymorphic merozoite/piroplasms surface protein (MPSP) of *Theileria orientalis* was amplified as per Sivakumar *et al*. (2012) (Fig 3).

Out of all positive blood samples for *Anaplasma marginale* and *Theileria orientalis* confirmed by PCR, two samples each from the above species were randomly selected for cloning and sequencing. After cloning, the clone was confirmed by colony PCR and sent for sequencing. The results of each sequence were annotated and submitted to NCBI GenBank for accession numbers of each sequence. Accession numbers for each sequence obtained from GenBank are given in the Table 1.

Phylogenetic analysis of partial *rpoB* gene segments of two isolates of *Anaplasma marginale i.e.* Miz-60 and Miz-61 (Fig 4) was carried out using reference sequences retrieved from the NCBI GenBank nucleotide database (Table 2). Comparative analysis was performed by use of the nucleotide sequences of *rpoB* genes of Mizoram state of India with other published sequences showing percent homology and divergence (Fig 5). The sequence of *rpoB gene* of two isolates from Mizoram states of India when compared with reference sequences, it was revealed that both the isolates were placed in same clade in the phylogenetic tree. These two isolates also showed close similarity with the other sequences of the same clade reported from different countries. This indicates the authenticity of our PCR-amplified *rpoB gene.* Nucleotide percent identity revealed both sequences of the isolates in the present study were 99.2% similar to each other. Percent identity as compared with other species of *Anaplasma*, revealing the lowest similarity *i.e.* 27.3% with Candidatus *Anaplasma africae* strain followed by 28.7% with *Anaplasma platys*, 29.5% with *Anaplasma centrale*, 33.5% with *Anaplasma ovis* and 80.2% with *Anaplasma phagoytophilum.* The isolates showed 94.6% similarity with the *Anaplasma marginale* obtained from *Rhipicephalus*

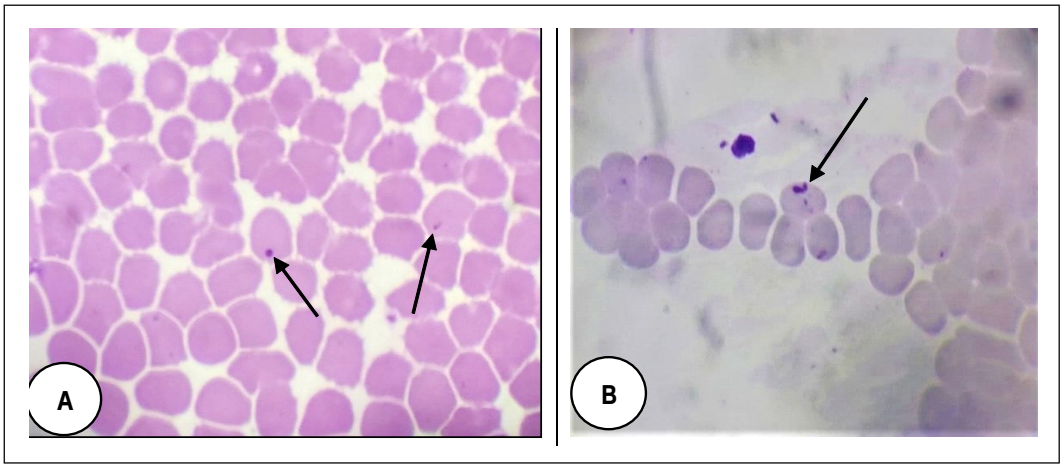


**Fig 1A:** Presence of *Anaplasma marginale* (Black Arrow). B: Presence of *Theileria sp.* (Black Arrow).

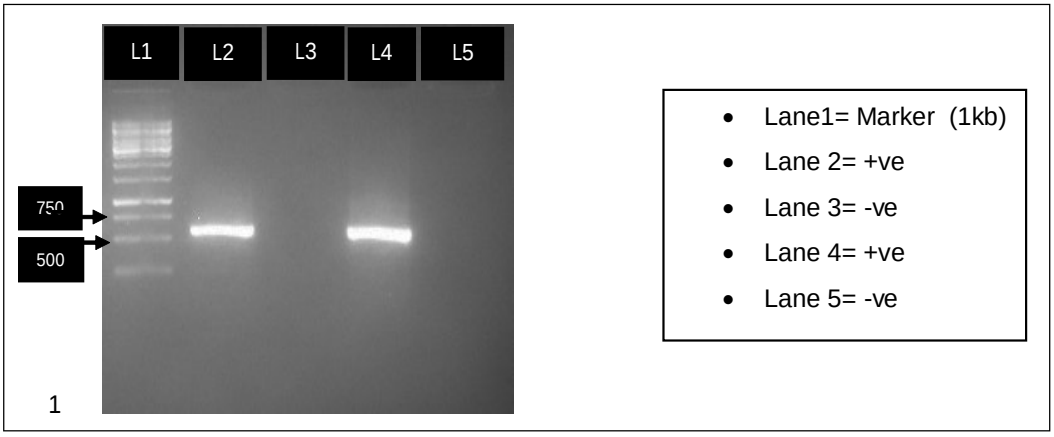


**Fig 2:** 1.2% Agarose gel electrophoresis stained with Ethidium bromide showing the 576 bp rpoB gene fragment of *Anaplasma marginale* in blood sample.

*bursa* (Cattle tick). The rpoB gene plays an important role in better differentiation between the closest species of Anaplasma with more sequence variations (Dahmani *et al.,* 2017). Das *et al.* (2022) also confirmed the *Anaplasma marginale* infections in cattle in Mizoram, India by taking the rpoB gene. Rangapura *et al*. (2019) reported on the

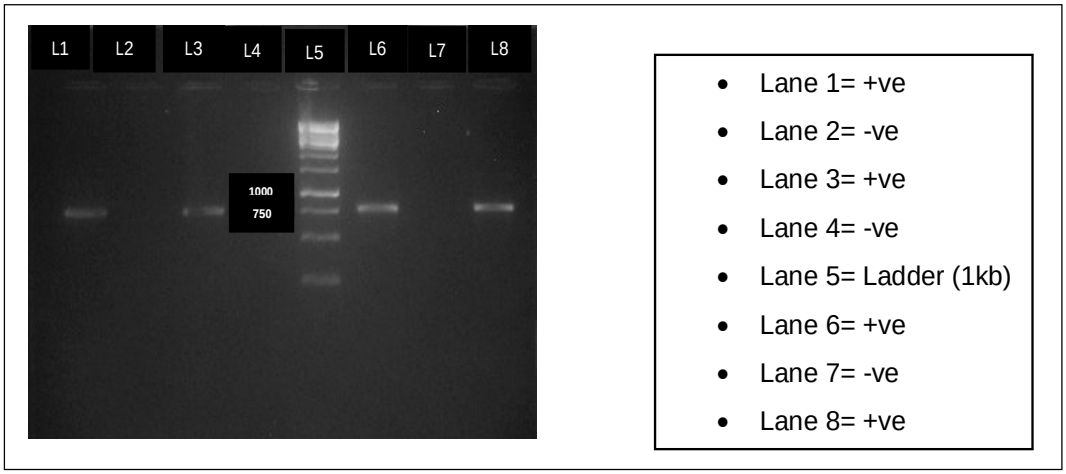


**Fig 3:** 1.2% Agarose gel electrophoresis stained with Ethidium bromide showing 776 bp gene encoding MPSP of *Theileria sp.* in blood sample.

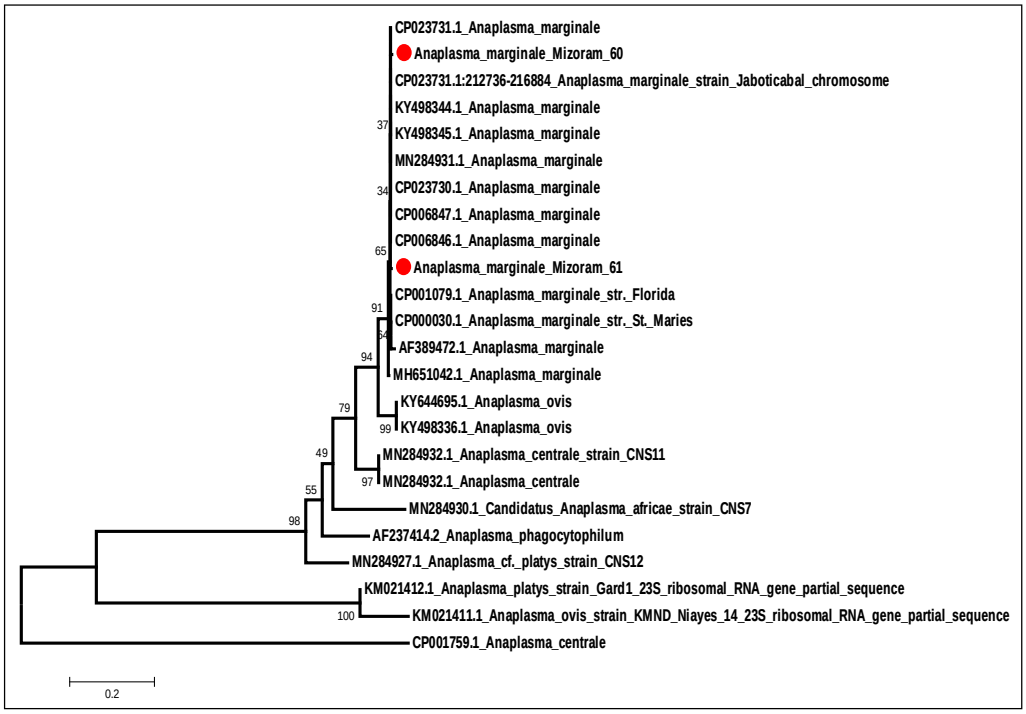


**Fig 4:** Phylogenetic tree illustrating among sequence based on RpoB gene of *Anaplasma sp.* sequences are taken from present study (red marked) and from GenBank.

**Table 1:** NCBI GenBank Accession number for nucleotide sequence of rpoB and MPSP genes.

| Organism | Lab. Isolate No. | Gene | NCBI Accession no. |
|---|---|---|---|
| *Anaplasma marginale* | Miz-60 | rpoB | MW057765 |
| *Anaplasma marginale* | Miz-61 | rpoB | MW057766 |
| *Theileria orientalis* | Miz-86 | MPSP | MW057767 |
| *Theileria orientalis* | Miz-87 | MPSP | MW057768 |

**Table 2:** List of reference sequences retrieved from NCBI website for Phylogenetic analysis of rpoB gene sequences of *Anaplasma sp.*

| Accession No. | Organism | Host | Country | Year |
|---|---|---|---|---|
| CP023731 | *A. marginale* | Cattle | Brazil | 2000 |
| CP023731:212736-216884 | *A. marginale* | Cattle | Brazil | 2000 |
| KY498344 | *A. marginale* | R. bursa (Cattle tick) | France | 2017 |
| KY498345 | *A. marginale* | R. bursa | France | 2017 |
| MN284931 | *A. marginale* | Cattle | Senegal | 2019 |
| CP023730 | *A. marginale* | Cattle | Brazil | 2000 |
| CP006847 | *A. marginale* | Cattle | Mexico | 2014 |
| CP006846 | *A. marginale* | Cattle | Mexico | 2014 |
| CP001079 | *A. marginale* | Cattle | USA | 2014 |
| CP000030 | *A. marginale* | Cattle | USA | 2014 |
| AF389472 | *A. marginale* | Cattle | France | 2016 |
| MN651042 | *A. marginale* | Rabbit | Portugal | 2019 |
| KY644695 | *A. ovis* | Sheep | Nigeria | 2018 |
| KY498336 | *A. ovis* | Sheep | France | 2017 |
| MN284932 | *A. centrale* | Cattle | Senegal | 2019 |
| MN284932 | *A. centrale* | Cattle | Senegal | 2019 |
| MN284930 | *Candidatus A. africae* | Cattle | Senegal | 2019 |
| AF237414 | *A. phagoytophilum* | White-tailed Deer | France | 2016 |
| MN284927 | *A. platys* | Cattle | Senegal | 2019 |
| KM021412 | *A. platys* | Dog | France | 2014 |
| KM021411 | *A. ovis* | Sheep | Senegal | 2014 |
| CP001759 | *A. centrale* | Cattle | USA | 2014 |

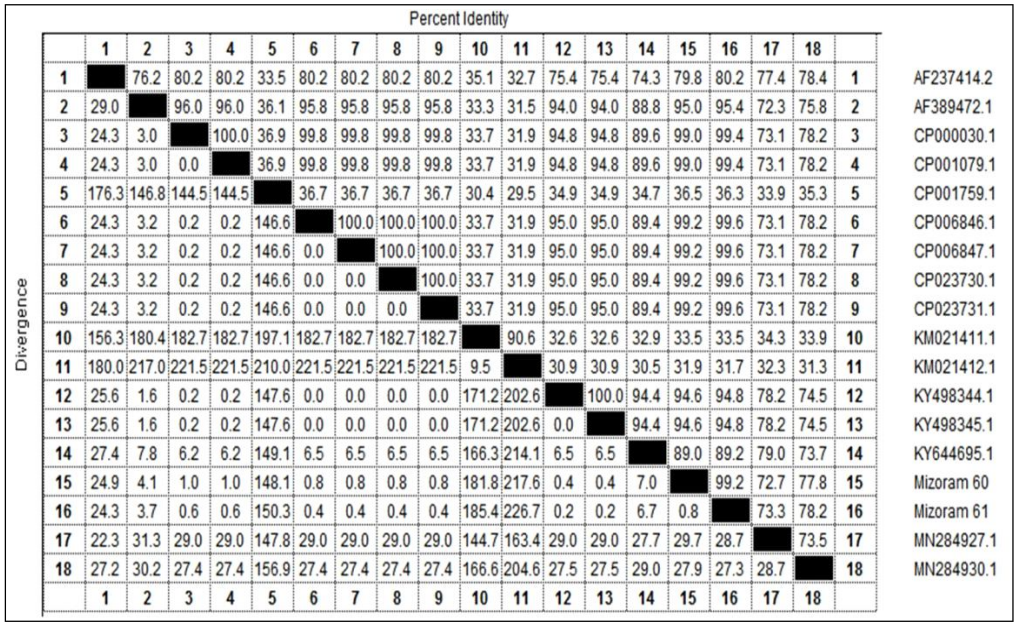


**Fig 5:** Comparative analysis of the nucleotide sequences of rpoB genes of Mizoram state of India with other published sequences showing percent homology and divergence.

molecular characterization of *Anaplasma sp.* in South India by taking the *16S rRNA* gene and revealed about the existence of *A. marginale, A. bovis and A. platys* infections in bovines. Other reports of molecular characterization of *MSP1,* a gene of *Anaplasma marginale* in different countries like North and South America, Asia, Africa, Australia and Europe revealed the genetic diversity of organisms (de la Fuente *et al.,* 2004).

Phylogenetic analysis was carried out for *Theileria sp*. Using MPSP partial gene fragment (Fig 6) of the present two isolates when compared with other reference sequences retrieved from GenBank (Table 3).Comparative analysis was

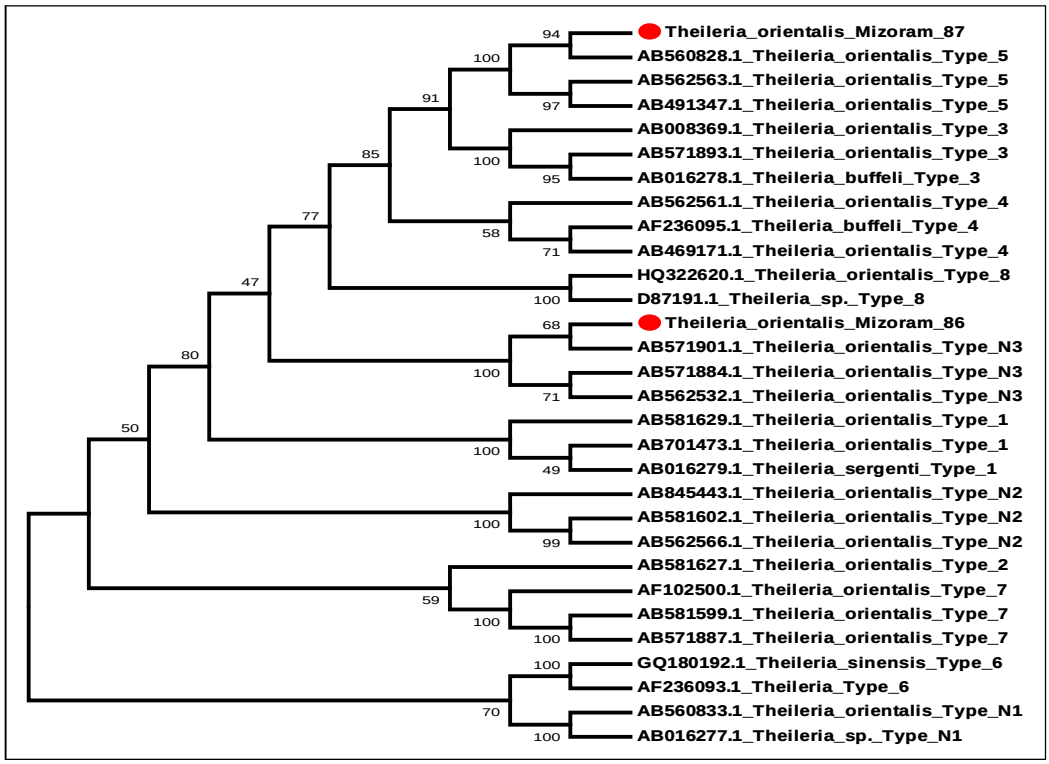


**Fig 6:** Phylogenetic tree illustrating among sequence based on MPSP gene *Theileria orientalis*. Sequences are taken from present study (red marked) and from GenBank.

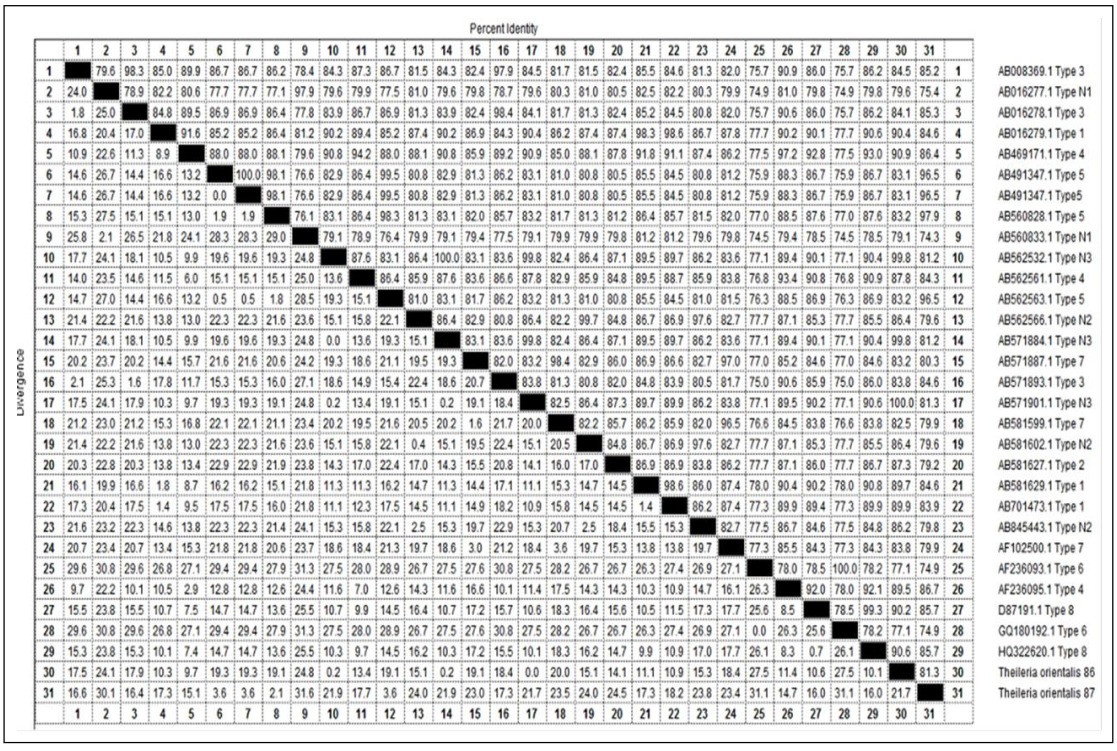


**Fig 7:** Comparative analysis of the nucleotide sequences of MPSP genes of Mizoram state of India with other published sequences showing percent homology and divergence.

**Table 3:** List of reference sequences retrieved from NCBI website for Phylogenetic analysis of MPSP gene sequences of *Theileria sp.*

| Accession no. | Organism | Host | Country | Year |
|---|---|---|---|---|
| AB560828 | *T. orientalis* | Water Buffalo | Vietnam | 2010 |
| AB562563 | *T. orientalis* | Water Buffalo | Thailand | 2010 |
| AB491347 | *T. orientalis* | Cattle | Japan | 2007 |
| AB008369 | *T. orientalis* | Cattle | Japan | 2000 |
| AB571893 | *T. orientalis* | Cattle | Mongolia | 2010 |
| AB016278 | *T. buffeli* | Cattle | Kenya | 2015 |
| AB562561 | *T. orientalis* | Water Buffalo | Thailand | 2010 |
| AF236095 | *T. buffeli* | Cattle | Australia | 2000 |
| AB469171 | *T. orientalis* | Cattle | Japan | 2007 |
| HQ322620 | *T. orientalis* | Cattle | Japan | 2011 |
| D87191 | *Theileria sp.* | Cattle | Korea | 2007 |
| AB571901 | *T. orientalis* | Cattle | Mongolia | 2010 |
| AB571884 | *T. orientalis* | *Rhipicephalus sp.* | Vietnam | 2010 |
| AB562532 | *T. orientalis* | Cattle | Thailand | 2009 |
| AB581629 | *T. orientalis* | Cattle | Brazil | 2009 |
| AB701473 | *T. orientalis* | Cattle | Sri Lanka | 2011 |
| AB016279 | *T. sergenti* | Buffalo | Russia | 2000 |
| AB845443 | *T. orientalis* | Buffalo | Sri Lanka | 2013 |
| AB581602 | *T. orientalis* | Cattle | Brazil | 2009 |
| AB562566 | *T. orientalis* | Water Buffalo | Thailand | 2010 |
| AB581627 | *T. orientalis* | Cattle | Brazil | 2009 |
| AF102500 | *T. orientalis* | Cattle | Indonesia | 2016 |
| AB581599 | *T. orientalis* | Cattle | Brazil | 2009 |
| AB571887 | *T. orientalis* | Cattle | Mongolia | 2010 |
| GQ180192 | *T. sinensis* | Yak | China | 2010 |
| AF236093 | *Theileria sp.* | Cattle | China | 2004 |
| AB560833 | *T. orientalis* | Sheep | Vietnam | 2010 |
| AB016277 | *Theileria sp.* | Buffalo | Vietnam | 2000 |

performed by use of the nucleotide sequences of MPSP genes of Mizoram state of India with other published sequences showing percent homology and divergence (Fig 7). From phylogenetic tree, it was revealed that the isolate *i.e.* Miz-86 was closely placed with Type-N3 clade. Whereas the other isolate *i.e.* Miz-87 belonged to Type-5 clade. Distinct variation in both types was further confirmed by only a sequence similarity of 81.3% among the isolates. When Miz-86 isolates were compared with the other reference sequence of Type-N3 revealed a similarity of 99.8 to 100%. Likewise, when the Miz-87 isolate was compared with the other reference sequences of Type-5, a similarity of 96.5 to 97.9% was observed. This confirms the detection of two genetic types of *Theileria orientalis* in Mizoram. Miz-86 isolates which are in Clade of Type-N3 showed more similarity with Type-4 other (90.9%) and the least similarity with Type-6 (77.1%). Type-N3 isolates were also identified from cattle and buffalo in Mongolia, Thailand, Vietnam and Brazil. Type-5 isolates were reported from cattle, water buffalo and cattle ticks in Japan, China, Korea, Thailand, Vietnam, Mongolia, Brazil and Sri Lanka (Sivakumar *et al*., 2012). The similarity with other types are 90.6%, 90.4%, 87.3%, 86.4%, 84.5%, 83.8%, 83.2% and 79.6% with Type-8, Type-1, Type-2, Type-N2, Type-3, Type-7, Type-5 and Type-N1 respectively. Miz-87 isolates which are in Clade of Type-5 showed more similarity with Type-4 other (86.7%) and least similarity with Type-N1 (74.3%). The similarity with other types are 85.7%, 85.3%, 84.6%, 81.3%, 80.3%, 79.8%, 79.2% and 74.9% with Type-8, Type-3, Type-1, Type-N3, Type-7, Type-N2, Type-2 and Type-6 respectively. Previously, Aparna *et al.* (2011) also detected *MPSP*-type 7 in clinical theileriosis in the southern part of India. Sivakumar *et al*. (2012) revealed the different types of genotypic distribution among different animals as well as in different geographical regions. There was a distribution of Type- 6 in cattle and yaks not in buffalo whereas Type N1 in water buffalo (Liu *et al.,* 2010; Sarataphan *et al.,* 2003). The predominance of Type-2 and 8 is mainly found in cattle populations (Kang *et al.,* 2012; Perera *et al.,* 2013; Yokoyama *et al.,* 2011). There are several genotypes of *Theileria orientalis* observed in bovines. Cross-infections also observed among the bovine species which may be due to differences in transmission of vectors.

## CONCLUSION

Studies for the presence of haemoprotozoan infections showed the presence of *Theileria sp.* and *Anaplasma sp.* Most of them were seen as subclinical infections except for

some clinical cases of Anaplasmosis. Cloning and sequencing of *Anaplasma* isolates indicate the authenticity of our PCR-amplified *rpoB gene*. Nucleotide identity revealed both sequences of the isolates in the present study were 99.2% similar to each other. The phylogenetic analysis of the isolate of *Theileria* i.e. Miz-86 was closely placed with the Type-N3 clade. Whereas the other isolate *i.e.* Miz-87 belonged to Type-5 clade. Distinct variation in both types was further confirmed by only a sequence similarity of 81.3% among the isolates.

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### Conflict of interest

All the authors declare that they have no conflict of interest regarding this present research work.

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