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Original article

Incidence of cutaneous habronemosis in Manipuri ponies in India

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ABSTRACT

Information pertaining to parasitic fauna and parasitic diseases in Manipuri ponies in India is not available. Moreover, no systematic studies have been undertaken on cutaneous habronemosis in Manipuri ponies which is a common skin problem of Manipuri ponies as reported by pony owners. Keeping in the view of the importance of parasitic infections in veterinary health coverage particularly in Manipuri ponies, the present study was planned. A survey of natural cases of cutaneous habronemosis followed by molecular confirmation of species involved and treatments were done. Out of 200 ponies examined, nine cases (4.5%) of cutaneous habronemosis was recorded. Gross examination revealed raised and ulcerated wounds with necrotic tissues covered with yellowish-tan granulation. Histopathological study revealed eosinophilic granuloma and in the center of the granuloma with necrotic debris. Remnants of the *Hebronema* larvae with infiltrating neutrophils surrounded by proliferating fibrous tissue with numerous eosinophils, macrophages and lymphocytes were also observed. Molecular detection of *Habronema* sp. was confirmed by semi-nested PCR. Sequence analysis revealed larvae of *H. muscae* was the common spirurid species responsible for producing cutaneous habronemosis in Manipuri ponies. Subsequently, sequence submitted to NCBI GenBank and accession number obtained (MH038181). Surgical removal of necrotic tissue, ivermectin injection along with antibiotics successfully cured all the lesions in infected ponies.Results confirmed occurrence of cutaneous habronemosis in Manipuri ponies in India.

1. Introduction

Habronemosis is an infection caused by spirurid nematode *Habronema*, where mostly three species are involved viz., *Habronema muscae, Habronema microstoma and Habronema megastoma or Draschia megastoma.* These worms do not affect cattle, sheep, goats, pigs, but sometimes affect dogs (Sanderson and Niyo, 1990). Predilection site of adult *Habronema* worm is the stomach of the horse. Larvae can be found in skin, eyes (Ali et al., 2016), genitalia (Pusterla et al., 2003) and even in the lungs (Schuster et al., 2010). All *Habronema* species have an indirect life cycle, with several fly species acting as intermediate hosts, mainly house flies i.e. *Musca domestica* or stable fly *Stomoxys calcitrans.* The infective L3 larvae migrate to the mouth of the flies from where they are deposited on the final host (horses, donkeys, etc.) visited by the flies. Once on the final host, L3 larvae are swallowed and get into the stomach where they complete development to adult worms. Infective L3-larvae are often deposited on humid parts of the host's body or on skin wounds producing skin or cutaneous habronemosis, also known as "summer sores" or *'bursati'*. The development of summer sore in the horse due to *Habronema* was first reported by Bull (1919) and Saceghem van (1919).

India holds about 0.63 million horses and ponies which are unevenly distributed all over the country (19th livestock census, 2012). So far, in India no specific literary information on cutaneous habronemosis in horses is available, but the occurrence of adult *Habronema* sp. in horses has been reported on the basis of examination of faecal samples (Pilania et al., 2013; Kachhawa et al., 2015). Recently, occurrence of bilateral conjuctival habronemosis in a Marwari horse has been reported from Rajasthan,India (Prasad et al., 2017). On the other hand, cutaneous habronemosis have been reported from many countries of Africa (Mohamed et al., 1989; Ali et al., 2016), USA (Pusterla et al., 2003; Valentine, 2005) and Europe (Traversa et al., 2004; Traversa et al., 2007; Verhaar et al., 2018).

Documentation of parasitic infestations of equines in our country is lacking. The state Manipur (a state in North Eastern India) has its own breed of Manipuri Pony. A survey carried out by the state's veterinary department in 2003 recorded pony population of 1893. The number came down to 1218 in 2007 and again it decreased to 1101 in 2012 (9th

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Manipur Polo International Souvenir, 2015). The Manipuri pony is an endangered breed of horse because it's population is decreasing alarmingly. Recently, we could record occurrence of different helminth species in Manipuri ponies and few cases were recorded with chronic granulomatous skin lesions (habronemosis?) and this is the background of the objective of this report.

2. Materials and methods

2.1. Selection of study area

The study materials were collected from ponies reared in Manipur Horse Riding and Polo Association, Lamphel (Imphal West district) and its surrounding areas from November 2016 to April 2017. The laboratory experiments were conducted in the Department of Parasitology, College of Veterinary Sciences & Animal Husbandry, Selesih, Aizawl, and Mizoram.

2.2. Selection of ponies

A total of 200 ponies, above 2 years of age, were selected randomly irrespective of health and management condition (84 males and 116 female) for screening of skin lesion/s. Faecal, as well as blood samples were collected fromall these ponies for evaluation of parasitic load and related diseases. A standard size of a Manipuri ponies is shown in Fig. 1.

2.3. Sampling procedure

Fresh faecal samples were collected in plastic sachets directly from rectum, wherever possible, and mid portion of the faecal pad from the floor using disposable polythene gloves. After labeling properly the collected samples were preserved at 4 °C. About 100 g of faecal materials were collected and divided into two parts, one part preserved in 70% Ethanol and the other part without any preservative i.e. freshly in sterile plastic bags and transported to the laboratory in the Department of Parasitology, College of Veterinary Sciences & A.H., CAU, Selesih, Aizawl for further process. Qualitative faecal examination was carried out by direct microscopic method, as well as, sedimentation and flotation methods as per procedure described by Hendrix (1998). Identification of eggs/ova was done according to morphological features of L1 (Soulsby, 1965).Extraction of the DNA was done from positive samples followed by Gene JET Genomic DNA Purification kit for further molecular detection. Cutaneous habronemosis condition was diagnosed on the basis of clinical appearance and symptoms (Dunn, 1969). Blood sample, faecal sample and biopsy materials were collected for further study from individual cases.

Fig. 1. Manipuri pony.

2.4. Collection of biopsy material

Tissue samples were collected aseptically from the wounds suspected to be infected with cutaneous habronomiosis. The tissue samples were processed for histopathological section and extraction of DNA for determining the actual species of *Habronema* larvae responsible for producing these lesion/s. About 10 g of tissue sample were collected from each of the lesion of infected animal with the help of sterilized scalpel. The collected tissue samples were put into the collecting tube separately in 70% ethanol, properly labelled and sealed, were bought to the laboratory for further examination.

2.5. Histopathology

Twelve tissue samples were collected from nine infected ponies and histopathological examination was done in the laboratory of the Department of Pathology, College of Veterinary Sciences & Animal Husbandry, Selesih, Aizawl, Mizoram following standard procedure (Luna, 1968).

2.6. Molecular detection of Habronema species

All the faecal sample positive for *Habronema species*ova were processed separately for the confirmation by molecular methods. About 5 g of faecal samples were homogenized in 15 ml of saturated salt solution using mortar and pestle. The samples were mixed thoroughly and strained with a strainer. Then, the samples were centrifuged at 2000 rpm for 5 min. About 2 ml of the supernatant was collected and mixed with 50 ml double distilled water in 50 ml centrifuge tube and centrifuged again for 5 min at 2000 rpm and repeated the steps for 4–5 times. The final washing was done twice in micro-centrifuge tube using nuclease free water and subsequently, Genomic DNA were extracted by using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific) and stored at −20 °C for further use. Whole genomic DNA was isolated by using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific) following manufacture's protocol from the skin lesions and the *Habronema* ova/ larvae obtained from faecal samples. The DNA samples obtained from direct faeces, copro-culture larvae and tissue/biopsy materials were subjected to two step semi nested PCR, using *Habronema* species specific primers as forward: D: 5′-GAGTCGATGAAGAACG CAG-3′and reverse: B1: 5 GAATCCTGGTTAGTTTCTTTTCCT-3′ (Traversa et al., 2004). Briefly, for primary PCR reaction mixture comprised 0.13 µL Taq polymerase, 0.25 µL dNTP (10 mM), 2.50 µl 10× Buffer (15 mM MgCl2), 0.75 µL of each forward (D) and reverse (B1) primer (60pM), and 1.0 µL template DNA (60 ng/µL concentration),by making the final volume to 25.0 µL with nuclease-free water.The assembled 25 µl reactions was incubated in thermal cycler (BioRad, C1000) following initial denaturation at 94 °C for 10 min and 30 cycles of denaturation at 94 °C for 30 s, an annealing temp of 58 °C for 45 s and extension of 72 °C for 45 s followed by final extension at 72 °C for 7 min. The PCR products were resolved at 1.5% agarose gel stained with ethidium bromide and photograph was taken using gel documentation system. Subsequently, by using the primary PCR product as DNA template a semi-nested PCR for species specificamplification was set up. In secondary PCR, forward primer of either Hmi (5′-GATCGCAATATGTGTAACAC-3′) for *H. microstoma* or Hmu (5′-CTG GTAAAGCATCAAT GCATCAGGTATG-3′) for *H. muscae* was used in combination with common reverse primer B1. PCR cycle was performed as initial denaturation at 94 °C for 10mins and 30 cycles of denaturation at 94 °C for 30 s, annealing temp at 58 °C for 45 s and extension of 72 °C for 45 s followed by final extension at 72 °Cfor 7 mins.The confirmation of the amplified products was made by gel electrophoresis of the PCR product in 1.5% agarose gel stained with ethidium bromide and visualized under gel doc (BioRad EZ Imazer). The specificity of the PCR amplification for the corresponding *H.muscae* target, both on representative positive larva samples and on biopsy sample was assessed

by amplicon purification followed by cloning and sequencing.

2.7. Cloning of ITS ribosomal DNA of Habronema muscae

Cloning of the PCR amplicon(s) for the genomic region as described above has been performed using pDrive cloning vector (Qiagen PCR Cloning Kit). DH5-alpha *E. coli* cell was used for transformation of the plasmid performed by heat shock method (Sambrook et al., 1989).The transformed cells were then plated immediately on pre-warmed LB agar plates supplemented with ampicillin (100 µg/ml), X-gal (30 µg/ml) and IPTG (0.5 mM/ml) for the development of blue and white colonies. Subsequently, clones were confirmed by clones'confirmation PCR. The clone of bacteria containing positive insert was stab cultured in LB agar in micro-centrifuge tube and sent for custom sequencing at the Department of Biochemistry, University of Delhi, South Campus. The sequence obtained was aligned and compared with other published sequence of *Habronema muscae* using Clustal W and BLAST. The obtained sequence was submitted to NCBI GenBank for accession number.

2.8. Treatment of clinically infected animals

All the clinically infected animals were kept separately inside the stall under supervision and treated accordingly. The treatment was done as follows: ivermectin (ivomac(R); Boehringer Ingelheim) injectionin the affected tissue at the dose rate of 200 µg per kg body weight on 0, 7, and 15th day, amoxicillin + clavulanic acid (Moxikind-CV(R); Mankind Pharma) at the dose rate of 3 g IM injection per day for 5 consecutive days and isoflupredone acetate (Isoflud(R);Zydus Animal Health) injection at the dose rate of 3 ml (2 mg per ml) on every alternate day for five occasions. During the clinical management periods fly repellants like Skin Heal (Herbal spray) were appliedin and around the lesions. Subsequently, response to the above treatments were observed and recorded.

3. Results

3.1. Incidence and pathology

Faecal examination of 200 ponies revealed presence of *Habronema* ova in 17 ponies (Fig. 2). There was no sex difference towards occurrence of the parasite between male and female horses. Of 17 ponies found harbouring *Habronema spp* 9 ponies (4.5%) were found infected with cutaneous habronemosis. Since, the incidence was recorded during six months period no seasonal variation/s could be ascertained. The lesions were mainly seen in the legs and lower abdomen. Gross examination revealed raised and ulcerated wounds. The center of the ulcer was necrotic and covered with yellowish-tan granulation tissue. The margins of the ulcer were round (Fig. 3(a)–(d). On cut sections, the masses were friable, and yellow-white caseous or gritty material was felt.Biopsy materials were collected and processed for histopathological examination at the Department of Pathology, College of Veterinary Sciences & Animal Husbandry, Selesih. Microscopic examination of the biopsy materials collected from cutaneous lesion revealed eosinophilic granuloma. The center of the granuloma contained necrotic debris and often remnants of the *Habronema* larvae with infiltrating neutrophils surrounded by proliferating fibrous tissue with numerous eosinophils, macrophages and lymphocytes. The proliferating tissue was highly vascularized and contained numerous newly formed blood vessels (Fig. 3(e)–(h)).

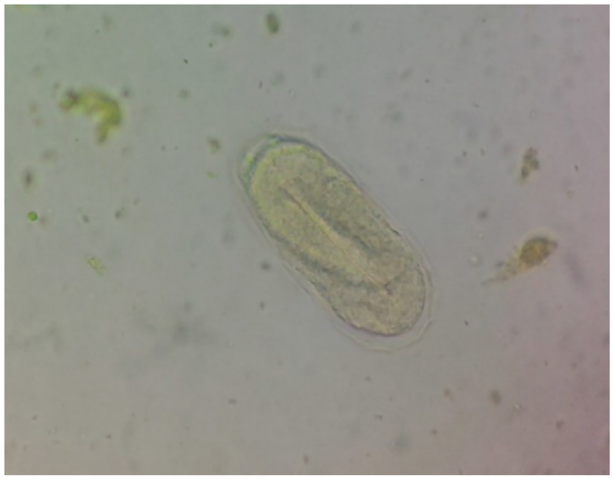

Fig. 2. Ova of *Habronema spp.*

3.2. Molecular detection of Habronema species involved in cutaneous habronemosis

DNA obtained from the faecal sample positive for *Habronema* egg, copro-cultured larvae and tissue/biopsy samples were processed for the two step semi-nested PCR amplification of ITS2 ribosomal DNA. The external primers (primer D forward and primer B1 reverse) showed *Habronema* genus specific amplification. The first step PCR amplification yielded ~ 546 bp of ribosomal DNA (internal transcribe spacer) in all the three-sample source. The primary PCR product was then subjected to amplification with species specific internal forward primers viz. Hmu and Hmi, for *H. muscae* and *H. microstoma*, respectively in combination with primer B1 as common reverse primer. The secondary PCR product by Hmu forward primer showed amplification at ~395 bp denoting the species of the parasite could be *H. muscae.* While the PCR reaction containing Hmi forward primer for the amplification of *H. microstoma* did not show any visible DNA in agarose gel. Fig. 4(a) and b. DNA sequence after custom sequencing was analyzed by using ClustalW and BLAST (NCBI). Subsequently, sequence submitted to NCBI GenBank and accession number obtained (MH038181).It was observed that there were only two changes in the nucleotide composition when compared with AY251023 where transition of G to A at 126th position and transversion of G to C at 374th position (Fig. 5). The obtained DNA sequence after analysis showed 98.4% similarity with the sequence of *H. muscae* ribosomal DNA (AY251023) as presented in Fig. 6.

3.3. Treatment of clinically affected animals

All the clinically infected ponies diagnosed for cutaneous habronemosis, were kept under supervision and Treatment was done as described elsewhere.Surgical removal of the necrotic tissues anddressing of the wounds were done on every alternate day After second dose of ivermectin injection, infected areas showed gradual healing and became reddish in colour. At the end of the third dose, there was complete healing of the wound with deposition of fibrous tissue. After 3 months, there was complete recovery of the wounds which were evident with growth of hair in the healed areas (Fig. 3(i)–(o)).

4. Discussion

4.1. Incidence of gastric infestation

The incidence of parasitic infection in Manipuri ponies in the Imphal west district (Lamphel) of Manipur, India was studied for the first time. Faecal examination revealed 8.49% of Manipuri ponies were harbouring *Habronema* species- the stomach worm of horse. There was no significant difference between male and female ponies towards occurrence of this parasite ($p < .05$). In India, only few workers had reported occurrence of gastric habronemosis in horse. Pilania et al. (2013) reported prevalence of *Habronema* sp. from different parts of Rajasthan, India (4.54%) with an average epg of 89.20 ± 7.19. On the other hand, in abroad 13% infection in equine was found in Victoria,

Fig. 3. (a–d). Ulcerated wounds in the limbs of Manipuri ponies. (e)-3(f). Eosinophilic granuloma (e) with remnants of *Habronema* larvae at the center (f). H&E, 100×. (g). Wall of the eosinophilic granuloma formed by proliferating fibroblast along with infiltrating eosinophils, macrophages and lymphocytes. (H&E, 100×). (h). Highly vascularized tissue of the granuloma with numerous newly formed blood vessels, proliferating fibroblast with infiltrating eosinophils, neutrophils andlymhocytes. (H&E. 200×). (i). On 1st day (1stdose). (j). On 3rd day. (k). On 7th day (2nd dose). (l). On 10th day. (m). On 15th day (3rd dose). (n). on 45th day. (o). On 90th day post treatment.

Australia (Bucknell et al., 2000) and low level of infection of 1.72% from Northwest Iran (Tavassoli et al., 2016). Slivinska et al. (2016) reported prevalence of 30% habrnemiasis in equine from Poland. Seasonal variation in the prevalence was studied in Germany where there was significantly higher ($p < .05$) number of infection was seen during summer (39.0%) followed by autumn (34.8%), winter (36.5%) and in spring (18.7%) (Rehbein et al., 2013).

4.2. Cutaneous habronemosis

The cutaneous type of habronemosis, “summer sore”, is often seen in areas of the world with a tropical or temperate climate (Gasthuys et al., 2004; Giangaspero et al., 2005). Indeed, the clinical diagnosis of the equine summer sore is challenging, as a lesion can be easily confused with other parasitic infections (e.g. onchocercosis), mycosis (e.g. botryomycosis, actinomycosis, pythiosis) or neoplasms e.g. sarcoid and squamus cell carcinoma (Pusterla et al., 2003).In this view, the laboratory diagnosis is crucial to confirm to any suspected case of equine summer sores. Unfortunately, since *Habronema* live only for less than one month in the cutaneous tissue (Fadok and Mullowney, 1983) usually there are only few larvae in lesions as an effect of the response elicited in infected animals. Furthermore, in cutaneous habronemosis

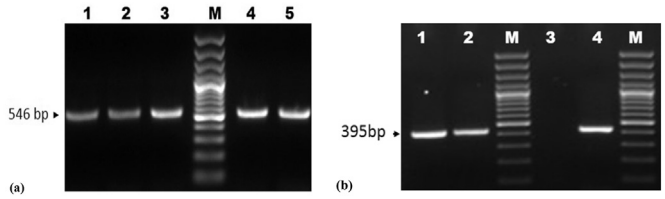

Fig. 4. (a). Primary PCR agarose gel showing *Habronema.*
Lane 1 & 2: DNA from faeces.
Lane 3: DNA from coproculture larvae.
M: Marker 100 bp.
Lane 4 & 5: DNA from biopsy/tissue.
(b) Secondary PCR agarose gel showing *Habronema muscae.*
Lane 1: DNA from faeces.
Lane 2: DNA from larvae.
Lane 3: Control.
Lane 4: DNA from biopsy.
M: Marker 100 bp.

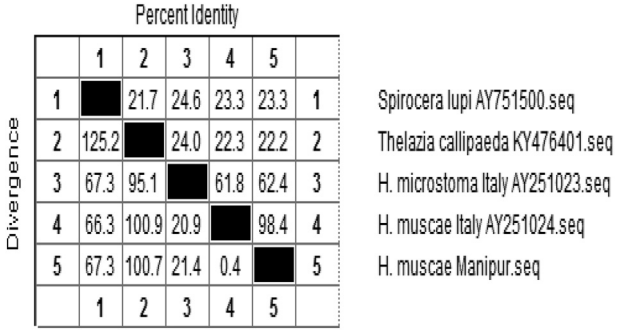

Fig. 6. Percent identity of *Habronema muscae.*

larvae death causes allergic immune response characterized by the appearance of ulcers, and exuberant, necrotic or calcified tissue (Soulsby, 1965) which greatly impairs the reliability of diagnosis by microscopical detection from skin biopsies, squeezes or smears (Dunn, 1969). Consequently, live *Habronema* larvae can only be traced in very early stages of skin lesions (Waddell, 1969).It is also known that the artificial digestion of skin samples is laborious and time consuming as the lysis reagent should be newly prepared immediately before performing the laboratory test by using fresh pepsin. Again, the direct microscopic larval detection requires specific technical skills to identify the larvae.

During the present studies nine Manipuri ponies were diagnosed for cutaneous habronemosis. The most common sites for cutaneous habronemosis were restricted to limbs and ventral abdomen of all the affected ponies. Lesions encountered were usually reddish-brown, with occasional ulceration. They often were “greasy,” sero-sanguineous, and contained yellow calcified “rice grain-like” materials. No sex predilection was noticed, since out of nine infected animals five male and four female ponies were recorded with cutaneous habronemosis. In a retrospective study from 1988 to 2002 out of 12,720 horses in United States, 63 cases were recorded with cutaneous habronemosis by Pusterla et al. (2003). They could record the lesions distributed on medial canthus of eye, male genitalia, and distal portion of extremities. They also further recorded 11 biopsy specimens positive for presence of nematode larvae during histopathology. In the present study histology section of one biopsy material revealed presence of larvae in cross section. Though faecal examination of Manipuri ponies revealed 8.5% habronemosis the prevalence of cutaneous lesions were less (4.5%). Similar type of results (5.6%) was recorded by Sharir et al. (1987) in Israel. In Western Europe, pathogenesis of cutaneous habronemosis is unknown but it is highly probable that the disease involves a hypersensitive reaction to dead or dying larvae because of the eosinophilia seen in direct smears and in histopathological sections. The lesions usually appear during spring and summer, probably related to a high fly activity, and regress in winter time. Although it is a rather sporadic disease, certain horses show an annual recurrence (Traversa et al., 2007).Spontaneous healing of cutaneous habronemosis in winter has also been recorded more recently in Netherlands (Verhaar et al.,

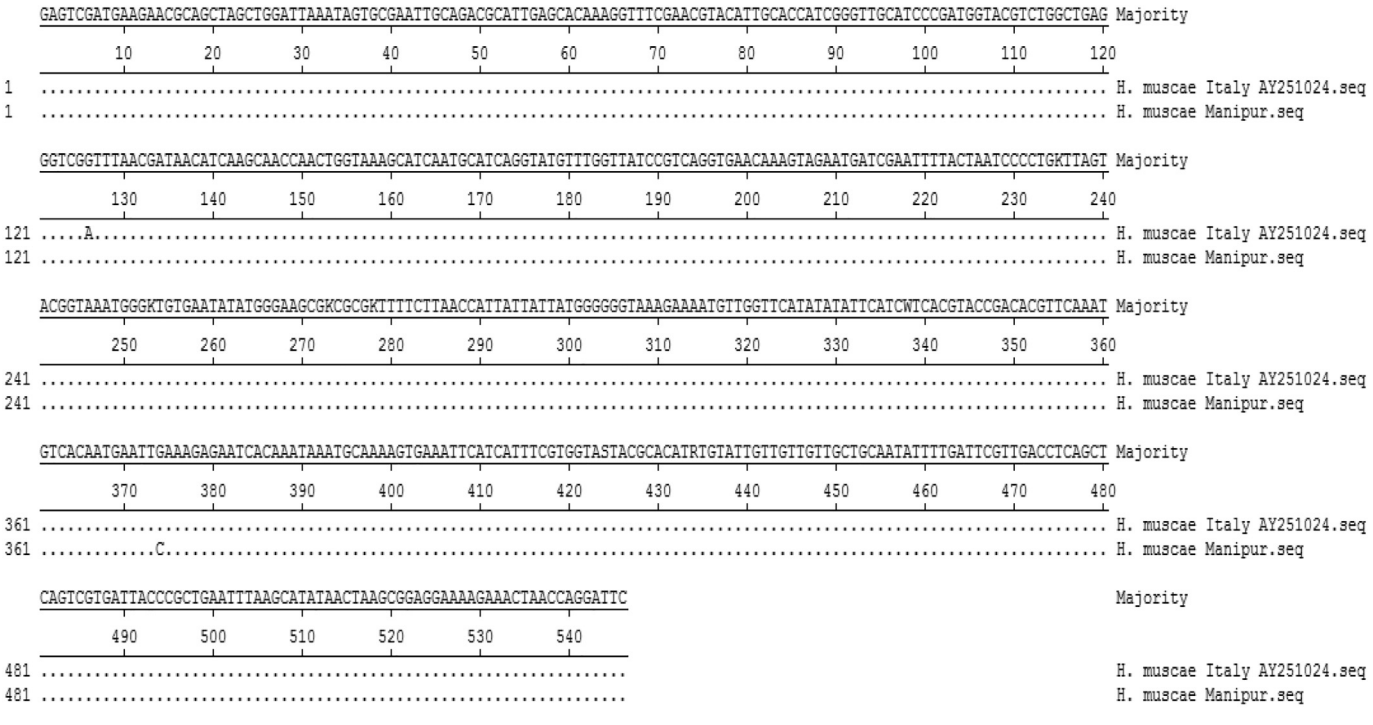

Fig. 5. Alignment report of ITS2 region of *Habronema muscae* between different isolates.

2018). Histopathological examination of a biopsy, although less sensitive, is currently the method of choice for confirming the diagnosis. Characteristic histological lesions consist of eosinophilic dermatitis and coagulative necrosis with or without degeneration of nematode larvae in the centre were seen in this study. This finding corroborated with the previous published reports (Pusterla et al., 2003; Traversa et al., 2007). Histo-pathological features of cutaneous habronemosis in equine have also been described by Amininajafi et al. (2014) from Tehran, Iran. Their observation of single or multiple ulcerated tumor masses with eosinophil epithelioid cell infiltration with necrotic debris in the lesions are similar with the findings with our present descriptions.

Besides, histological confirmation of cutaneous habronemosis in Manipuri ponies, a further confirmatory molecular diagnosis for actual larval involvement with the sore was also assessed. The present study yielded a positive amplification of DNA for *H. muscae* species under semi nested PCR technique at 395 bp corroborated with the findings of Traversa et al. (2007). The semi-nested PCR herein presented applied on skin samples open further avenues for the diagnosis and epidemiology of cutaneous habronemosis in Manipuri ponies. A molecular prompt diagnosis would allow a timely treatment, thus leading a successful recovery of the infected animals while a false negative diagnosis and untimely treatment would result in disfiguring infections. Many treatments for habronemosis have been reported, including corticosteroids for reducing the inflammatory hypersensitivity reactions and ivermectin to kill the larvae and adult worms in stomach (Pusterla et al., 2003).Topical combinations of anti-inflammatory, larvicidal and antimicrobial ingredients are also recommended (Pusterla et al., 2003; Giuliano, 2011). Treatment should be aimed at decreasing the size of lesion, reducing inflammation and preventing recurrence. Considering the severity of cutaneous lesions, all nine Manipuri ponies were treated with topical and systemic ivermectin combined with antibiotics and corticosteroid to kill the worms and control the secondary bacterial infections and inflammation. After three months of treatments all the three ponies were found complete healing of wounds.

5. Conclusion

In conclusion, the present studies confirms, that the cutaneous habronemosis occurs in Manipuri ponies. The recorded species involved was confirmed as larval stages of *Habronema. Muscae.* This is the first report of occurrence of cutaneous habronemosis in Manipuri ponies in India. Further, present studies described the successful treatment regime.

Authorship

Chirom Nishita Devi contributed to the data collection and analysis. Sonjoy Kumar Borthakur contributed to the study design and molecular analysis of data and interpretation. Gautam Patra, Shymsena Singh and Subhamoy Ghosh helped with analysis and R.Ravindran helped with histopathology of biopsy materials. All the authors contributed to the preparation of the paper and approved the manuscript for publication.

Authors' declaration of interests

No competing interests have been declared.

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