Molecular evidence suggests the occurrence of *Entamoeba moshkovskii* in pigs with zoonotic potential from eastern India

Sanjib K. Sardar1, Koushik Das1,2, Maimoon Maruf6, Tapas Haldar1, Yumiko Saito-Nakano1, Seiki Kobayashi1, Shanta Dutta3 and Sandipan Ganguly1,3,5

1 Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED), Kolkata, India
2 Department of Allied Health Sciences, School of Health Sciences, University of Petroleum and Energy Studies, Dehradun, India
3 Department of Parasitology, National Institute of Infectious Diseases (NIID), Tokyo, Japan
4 Department of Infectious Diseases, Keio University School of Medicine, Tokyo, Japan
5 Division of Parasitology, ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED), Kolkata, India

Abstract: *Entamoeba moshkovskii* Tshalaia, 1941 is prevalent in developing countries and it is considered to be primarily a free-living amoeba, which is morphologically indistinguishable, but biochemically and genetically different from the human infecting, pathogenic *Entamoeba histolytica* Schaudinn, 1903. The pathogenic potential of this organism is still under discussion. *Entamoeba moshkovskii* in human stool samples has been reported in different countries such as the United States, Italy, Australia, Iran, Turkey, Bangladesh, India (Pondicherry), Indonesia, Colombia, Malaysia, Tunisia, Tanzania and Brazil, but no data are available about the occurrence of *E. moshkovskii* in farm animals. This study provides data on the occurrence of *E. moshkovskii* in pigs in a total of 294 fresh faecal samples collected from five different regions in Kolkata, West Bengal, India. Stool samples were tested by nested PCR using primers targeting SSU rDNA of *E. moshkovskii*. The amplified PCR products were further confirmed by RFLP technique. Purified nested PCR products were also sequenced and identified via BLAST program run on the NCBI website to confirm species along with their genetic characteristics of the *E. moshkovskii* isolates. Overall 5.4% samples were identified as *E. moshkovskii* positive. Results of this study demonstrate that swine can host *E. moshkovskii* and should be considered as a potential natural reservoir for *E. moshkovskii*. However, the occurrence of *E. moshkovskii* infection in pigs was not statistically associated with their faecal consistency, sex and developmental stage.

Keywords: PCR, RFLP, host, natural reservoir, intestinal protists, diarrhea

The genus *Entamoeba* Casagrandi et Barbagallo, 1895 consists of at least nine species: *E. histolytica* Schaudinn, 1903, *E. dispar* Brumpt, 1925, *E. moshkovskii* Tshalaia, 1941, *E. bangladeshi* Royer, 2012, *E. coli* (Grassi, 1879), *E. nuttalli* (Catellani, 1908), *E. hartmanni* Prowazek, 1912, *E. polecki* Prowazek, 1912 and *E. chattoni* Levine, 1961, which are able to reside in the human intestinal lumen (Fotedar et al. 2007b, Delialioglu et al. 2008, Ngui et al. 2012). The first three species are the most prevalent and are morphologically similar under light microscope, but have different biochemical and genetic characteristics (Verweij et al. 2003). Although *E. polecki* has rarely been implicated as a cause of diarrheal disease, it is important to keep in mind that most of species are usually believed as commensal organisms of the human gut except *E. histolytica* and *E. moshkovskii* (see Salaki et al. 1979, Al-Areeqi et al. 2017). *Entamoeba histolytica* is considered as the most recognised pathogen of the human gut. Nonetheless, recent studies have reported the association of *E. moshkovskii* with gastrointestinal clinical manifestations indicating *E. moshkovskii* might be associated with pathogenicity (Ali et al. 2003, Fotedar et al 2007b, Khairnar and Parija 2007, Ngui et al. 2012). So far, the role of *E. moshkovskii* as an etiological agent of diarrhea in humans remains unclear (Clark and Diamond 1991).

*Entamoeba moshkovskii* was first described as a distinct species from Moscow by Tshalaia in 1941 (see Scaglia et al. 1983). It was primarily considered to be a free-living environmental strain *Entamoeba* sp. and is still regarded as a common protist species found in anoxic sediments and brackish coastal pools. It is osmotolerant in nature and can be cultured in various media suitable for intestinal protists, in which it grows easily at temperatures of 10–15°C and 37°C (Diamond and Bartgis 1970, Scaglia et al. 1983, Clark and Diamond 1991).

In 1961, an *E. histolytica*-like strain was obtained from a resident of Laredo, Texas, who suffered from diarrhea, weight loss and epigastric pain; this strain was named as...
E. histolytica Laredo strain as it shared many biological characteristics with E. moshkovskii (see Scaglia et al. 1983). Both the Laredo strain and E. moshkovskii grew easily at room temperature and were osmotolerant and resistant to drugs used in the chemotherapy of amoebiasis such as emetine (Fotedar et al. 2007a). Subsequent molecular studies revealed that the E. histolytica Laredo strain is actually a strain of E. moshkovskii; therefore, this strain was the first human isolate of E. moshkovskii.

Entamoeba moshkovskii in human stool samples has been detected in many countries such as the United States, Iran, Turkey, Italy, Australia, Bangladesh, India (Pondicherry), Indonesia, Colombia, Malaysia, Tunisia, Tanzania and Brazil, but no data are available about the occurrence of E. moshkovskii in farm animals. Although a recent study reported the presence of E. moshkovskii in non-human primates (NHP), data on the prevalence of E. moshkovskii in other animals are limited (Levecke et al. 2010). Therefore, the role of these animals as a potential reservoir for zoonotic transmission of E. moshkovskii remains unclear. Most of the previous studies were conducted for detection of cysts or trophozoites in human stools via light microscopy or PCR. As a result, the distribution and natural host range of E. moshkovskii are largely understudied.

Pai et al. (2003) reported the occurrence of E. histolytica/E. dispar on the cuticle and/or digestive tract of American cockroaches Periplaneta americana (Linnaeus) and in the digestive tract of German cockroach Blattella germanica (Linnaeus) in South Taiwan (Pai et al. 2003). Some non-human primates, cats and dogs have also been identified as natural hosts of E. histolytica (see Muruiki et al. 1998). Another study showed that two species, Entamoeba suis Hartmann, 1913 and E. polecki have been identified in pigs (Ji et al. 2019). Entamoeba suis is mostly restricted to pigs and causes hemorrhagic colitis by breaking down the lamina propria (Matsubayashi et al. 2014). Whereas E. polecki causes infection in many natural hosts, including humans, non-human primates and pigs, the epidemiology of its infections and particularly the role of non-human hosts as a potential reservoir for zoonotic transmission remain unclear (Muruiki et al. 1998; Tachibana et al. 2009).

In India swine husbandry is on the rise and it plays a crucial role in livestock farming. The risk of exposure of zoonotic parasitic agents such as Entamoeba spp. from pigs is thus predictable. In this study we report the occurrence of E. moshkovskii in stool samples from indigenous pig breeds farmed in and around Kolkata, West Bengal. This study suggests the potentiality of swine as a reservoir for E. moshkovskii that might be a route for its zoonotic transmission.

MATERIALS AND METHODS

Study area and population
A total of 294 fresh faecal samples were collected between February 2018 and December 2019 from five different regions in Kolkata, West Bengal, India. All specimens were collected from piglets (< 4 weeks old), weaners (1–3 months old), porkers (3–9 months old) and hogs (> 12 months old) immediately after defecation on the ground. From each of the animal pen one sample was collected.

Sample collection and microscopy
Faecal consistency, sex and developmental stage of pig were recorded at the spot of sample collection. Faecal samples were transported to the laboratory in ice boxes within 2 hours and preserved in at 4°C before microscopic analysis. After microscopy, DNA isolation was done generally within 48 hours of sample collection. A portion of each sample was examined microscopically in both saline wet mounts and Lugol’s iodine wet mounts for cysts of amoebae.

DNA extraction
Total genomic DNA was isolated directly from approximately 200 mg of each faecal sample that was found positive for cysts of Entamoeba spp. through microscopy, using STOOL DNA Minikit (QIAGEN, USA) as per manufacturer’s instructions. The isolated DNA was kept at -20°C until molecular identification of Entamoeba moshkovskii.

PCR amplification
For detection of the SSU-rDNA gene of E. moshkovskii in faecal DNA, a set of previously described nested primers was used: Em_F1 5’CTCTTACCGGGAGTGCG-3’, Em_R1 5’TGGTTTAATATACCTT-3’, Em_nF2 5’GAATAAGGATGTATGAC-3’ and Em_nR2 5’AAGTGGAGTTAACACCT-3’. Primary PCR for E. moshkovskii was performed in a final volume of 50 μl mixture 50 μl contained 1X PCR buffer, 4.0 mM MgCl₂, 0.2 mM of each dNTP, 0.1 M of each forward and reverse primer (Em_F1 & Em_R1), 1U of Taq polymerase (Bioline), 3 μl of stool DNA samples at 50 ng/μl concentration. The PCR cycle for primary PCR was as follows: 96°C for 5 minutes followed by 35 cycles each consisting of 94°C for 45 seconds, 60°C for 40 s and 72°C for 45 s, following by a final extension at 72°C for 7 min. Successively, 1.5 μl of primary PCR products was subjected as a template for the nested PCR. Amplification was achieved using as described above for primary PCR except MgCl₂ concentration and annealing temperature. MgCl₂ concentration was 1.5 mM and annealing temperature was 55°C for the nested PCR cycle. Nested PCR generated amplicons of 258 bp in presence of E. moshkovskii. Amplified PCR products after nested PCR were separated by agarose gel electrophoresis and visualised in a UV transilluminator after 0.5 μm/ml of ethidium bromide staining.

Restriction fragment length polymorphism (RFLP) analysis
The nested PCR products were confirmed by RFLP. The restriction enzyme MboII recognises the non-palindromic sequence 5’–GAAGA–3’/3’–CTTCT–5’ and cleaves DNA 8 and 7 nucleotides downstream of the recognition site in the 5’ to 3’ direction on the upper strand, leaving a single 3’ protruding nucleotide (Furmanek-Blaszk et al. 2009). This recognition site is present at 206/205 position in the amplicon of E. moshkovskii 18S rDNA locus. Around 120–160 ng of PCR products were digested with Mbo II (NEB) in a total volume of 20 μl reaction mixture at 37°C for 1 hour, followed by heat inactivation at 65°C for 20 min. Digested PCR products were separated by 1.8% agarose gel electrophoresis and visualised in a UV transilluminator after 0.5 μm/ml of ethidium bromide staining.
Table 1. Distribution of *Entamoeba moshkovskii* Tshalaia, 1941 in pigs according to their developmental stage, sex and faecal consistency.

| Variables            | No. examined | No. positive | Prevalence (%) | 95% CI     | X²  | p value | df |
|----------------------|--------------|--------------|----------------|------------|-----|---------|----|
| Developmental stage  |              |              |                |            |     |         |    |
| Piglets (< 4 weeks old) | 38           | 1            | 2.9%           | 0.1–13.5%  |     |         |    |
| Weaners (1–3 months old) | 68           | 3            | 4.4%           | 1.2–12.2%  | 3.43| 0.33    | 3  |
| Porks (3–9 months old) | 73           | 7            | 9.6%           | 4.7–18.5%  |     |         |    |
| Hogs (> 12 months old)/Adult | 115         | 5            | 4.3%           | 1.9–9.8%   |     |         |    |
| Sex                  |              |              |                |            |     |         |    |
| Male                 | 163          | 10           | 6.1%           | 3.4–10.92% | 0.341| 0.56   | 1  |
| Female               | 131          | 6            | 4.6%           | 2.1–9.6%   |     |         |    |
| Faecal consistency   |              |              |                |            |     |         |    |
| Soft faeces          | 94           | 5            | 5.3%           | 2.3–11.9%  | 0.013| 0.994  | 2  |
| Firm faeces          | 143          | 8            | 5.6%           | 2.9–10.7%  |     |         |    |
| Liquid faeces        | 57           | 3            | 5.3%           | 1.4–14.4%  |     |         |    |

CI – Confidence interval; X² – chi-square value; df – degrees of freedom

DNA sequencing

Positive PCR products were sequenced to test the specificity of the performed PCR assay. PCR products were purified using the Roche PCR Gel extraction Kit and sequenced with the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on a DNA analyser (Applied Biosystems, Foster City, CA, USA). The accuracy of the sequence was verified by using both forward and reverse primer separately during sequencing in 5’-3’ direction. The obtained sequences were aligned using ClustalW multiple sequence alignment program of GenomeNet Bioinformatics tools and edited manually. The obtained 18S rRNA gene sequences of *E. moshkovskii* were compared to those available in the GenBank database using the BLAST program run on the NCBI website.

Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura 1992). The tree with the highest log likelihood (-389.77) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1258)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00 % sites).

We opted four known sequences of *Entamoeba histolytica* (KJ870211.1), *Entamoeba dispar* (AB282661.1), *Entamoeba bangladeshi* (KR025412.1) and *Entamoeba nuttalli* (AB374947.1) to construct the phylogenetic tree of the *E. moshkovskii* isolates obtained in this study. These four species were chosen because they are the closest species of *E. moshkovskii*. This analysis involved 17 nucleotide sequences. There was a total of 222 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). The obtained nucleotide sequences in this study have been submitted to NCBI GenBank database.

Statistical analysis

GraphPad prism v.8.4.2, CA, USA was used to analyse the data. The relationship between the occurrence of *E. moshkovskii* with other variables like faecal consistency, age, sex and developmental stage were measured by testing X². In all cases differences were considered statistically significant when p-value was less than 0.05.

RESULTS

Occurrence of *Entamoeba moshkovskii* in pigs

Microscopic examination of 294 faecal samples revealed that 87.8% (n/N = 258/294, 95% CI 83.5–91.0%) contained cysts/trophozoites of *Entamoeba* spp. (Fig. 1). All the 258 microscopically positive samples were subjected to PCR for detection of *Entamoeba moshkovskii*. A total of 16 out of 258 samples were positive for *E. moshkovskii*, detected by nested PCR assay. Therefore, 6.2% (n/N = 16/258, 95% CI 3.8–9.9%) of all the *Entamoeba* spp. infected samples contained *E. moshkovskii* DNA. Overall, 5.4% (n/N = 16/294, 95% CI 3.4–8.7%) samples were detected as *E. moshkovskii* positive among all the samples collected.

Distribution of *E. moshkovskii* infection in pigs

The occurrence of *E. moshkovskii* infection in pigs was higher in males but the difference was not statistically significant (see Table 1). The association of *E. moshkovskii* infection in swine with faecal consistency was not statistically significant. It was also observed that the *E. moshkovskii* infection in pigs were not significantly associated with their developmental stage (Table 1). However, highest (9.6%, 95% CI 4.7%–18.5%) and lowest (2.6%, 95% CI 0.1%–13.5%) prevalence of infection of *E. moshkovskii* was found among porks and piglets, respectively. However, a specific seasonal pattern was not observed for *E. moshkovskii* infection in pigs (data not shown). The detailed data of distribution of *E. moshkovskii* with their statistical association are shown in the Table 1.

Rate of infection in different pig farms

A total of 88% (n/N= 14/16) of the specimens positive for *E. moshkovskii* were collected from the swine rearer in the geographical location of Kolkata Leather Complex area (22.4984 N; 88.5172 E).

Nested PCR-RFLP of 18S rDNA locus

After the amplified PCR products were digested with restriction endonuclease MboII, we detected the presence of *Entamoeba* spp. The RFLP pattern for *E. moshkovskii* showed 214 bp and 44 bp fragments. All (n = 16) of the
obtained amplicons were digested by the MboII restriction enzyme and produced RFLP pattern for *E. moshkovskii*.

**Phylogenetic analysis of *E. moshkovskii* isolates**

Among the 16 positive samples of *E. moshkovskii*, 18S rRNA gene sequences were obtained from 12 samples. Sequencing of the amplified PCR products demonstrated that they belonged to *E. moshkovskii*. The obtained sequence was deposited in NCBI GenBank database with accession number MW926950 and MZ357989–MZ357999 (18S rDNA). BLAST results showed that seven sequences displayed 100% sequence similarity to the *E. moshkovskii* Laredo strain (considered as a prototype) in NCBI Genbank (accession no. AF149906.1). DNA sequencing of four amplified products (MW926950, MZ357989, MZ357991, MZ357997 and MZ357897) showed that the sequence (258 bp) of this product was 99.46% identical to that of the corresponding reference sequence of *E. moshkovskii* (AF149906.1). The remaining sample (MZ357990) showed an identity of 99.22%. Only 1–2-bp nucleotide substitutions (448T/G, 487G/T) were identified. Both identified substitutions corresponded to transversion mutations (pyrimidine ↔ purine). The constructed phylogenetic tree revealed that MW926950, MZ357989, MZ357991, MZ357979 and MZ357997 were the closest variants of the reference isolate (MZ357998–MZ357993), whereas MZ357990 was the most distant sequence from the reference sequence but they belonged to same species. MZ357990 shared a common clade with the closest variants of the reference isolate (Fig. 3). The constructed Maximum Likelihood tree showed the similar results with PCR assay, i.e., they fitted in with *E. moshkovskii*. These results specify that the obtained *Entamoeba* isolates from swine stool samples in Kolkata were *E. moshkovskii*.

**DISCUSSION**

For examination of *Entamoeba* spp. in stool samples (fresh or fixed), traditional microscopy has always been the most commonly used diagnostic tool. Although this strategy has a clear downside, i.e., the fact that several species of *Entamoeba* are morphologically indistinguishable from each other via microscopy alone (e.g., *Entamoeba dispar*, a nonpathogenic species, is morphologically identical to *Entamoeba histolytica*). Therefore, to accurately identify the species/subtypes of *Entamoeba moshkovskii*, molecular tools including PCR and nucleotide sequencing had to be used (Verweij et al. 2003, Fotedar et al. 2007a, Fotedar et al. 2007b, Khairnar and Palaniappan 2007, Delialioglu et al. 2008, Ngui et al. 2012).

The present study successfully identified *E. moshkovskii* from swine stool samples using molecular techniques. A moderately high prevalence of *E. moshkovskii* infection in pigs was recorded in this study. Prevalence of *Entamoeba suis* and *Entamoeba polecki* in pigs was reported in different studies to be from 3.7% to 91.7% (Ji et al. 2019). However, the occurrence of *E. moshkovskii* infection in pigs/farm animals has not been recorded. The present study showed that 87.8% of samples were positive for *Entamoeba* spp., but only 5.4% of samples contained *E. moshkovskii* DNA. Sequencing of amplified PCR products revealed the presence of *E. moshkovskii* of four novel genotypes, whereas remaining eight samples were similar to previously described genotypes reported in humans. We observed that the *E. moshkovskii* infection rate in pig was

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**Fig. 1.** Microscopic view of cysts of *Entamoeba* spp. after staining.

**Fig. 2.** A – amplification of 18S rDNA locus of *Entamoeba moshkovskii* (Tshalaia, 1941) from pigs. PCR product size: 258bp. 1–13 are pig stool samples collected from Kolkata and adjacent areas; 14 – positive control containing *E. moshkovskii* DNA; 15 – negative control; lanes 3, 7, 8 and 10 are positive for *E. moshkovskii*; B – *Entamoeba moshkovskii*-specific nested 18S rDNA PCR product. a1, b1, c1, d1 and e1 are representative undigested nested PCR products of positive samples. a2, b2, c2, d2 and e2 are MboII digested PCR products. Pc1 is undigested PCR product of positive control product. Pc2 is MboII digested positive control PCR product.
not statistically associated with different variables like age, sex and stool consistency.

These results indicate that the infection occurred in sporadic manner. Ingestion of polluted water might be associated with differences in infection rate. Investigation of clinical importance of the infection in pig was not performed in depth. More research studies are needed in order to get better understanding of transmission, zoonotic potential and epidemiology of *E. moshkovskii* in pigs. Genotyping of *E. moshkovskii* isolates from swine in different geographical areas could be performed for additional confirmation.

This is the first study that reports the occurrence of *E. moshkovskii* in farm animals to the best of our knowledge. Amoebae of the genus *Entamoeba* are well known as parasitic species but many of them are free-living as well. *Entamoeba moshkovskii* is an amphizoic amoeba that can occur in environmental or endozoic conditions (Fotedar et al 2008, Clark and Diamond 1991). Until now, *E. moshkovskii* has been identified mostly in samples from treated waste water used for agriculture, sewage and human stool (Diamond and Bartgis 1970, Scaglia et al. 1983). Therefore, it can be transmitted between pigs by contact or ingestion of any unclean water sources. Although a previous study showed NHP (owl-faced monkey, *Cercopithecus hamlyni* Pocock. Javan lutung, *Trachypithecus auratus* Saint-Hilaire and Northern plains gray langur, *Semnopithecus entellus* Dufresne) as novel host species for *E. moshkovskii* (see Levecke et al. 2010).

In general, a limited study has been conducted to explore the distribution of *E. moshkovskii* in animals. It will be significant in future studies to focus on zoonotic transmission and pathogenic role of *E. moshkovskii* and to elucidate the host specificity of individual variants. Our study was conducted in five different locations and *E. moshkovskii* occurred in two of them, which indicates that the distribution of *E. moshkovskii* is not uniform in these animals.

This study may boost research for a better understanding of the nature of infection of *E. moshkovskii*. Moreover,
ver, some recent findings support the pathogenicity of *E. moshkovskii* in humans and show that this amoeba may have a similar host range as *E. histolytica* (see Ali et al. 2003, Parija and Khairnar 2005). Susceptibility of swine to *E. histolytica* was revealed in some experiments that indicated a role of swine as a reservoir for *E. moshkovskii*, which is closely related to *E. histolytica*. We speculate that the infection of swine with *E. moshkovskii* was through consumption of contaminated food or water and spread to other individuals via faecal-oral route. The possibility of transmission of the parasites from swine to humans cannot be ruled out. Although the present study was carried out in a fairly small number of samples, relatively common occurrence of *E. moshkovskii* in this study indicates that pigs are true hosts for this parasite and the occurrence of *E. moshkovskii* in pig warrants further attention.

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