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Studies on Occurrence of Trichinellosis in Pigs and Its Molecular Characterization Using Multiplex PCR in Maharashtra, India

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A B S T R A C T

Trichinellosis is important food-borne parasitic zoonoses caused by consumption of raw or under-cooked meat from a wide variety of wild and domestic mammals. Pork is consumed across the various pockets of India and it is most important source of infection to humans for Trichinellosis. Recent reports on presence of *Trichinella* spp. in pork sold in Maharashtra, India is concern for consumers. Therefore the study was planned to check occurrence of *Trichinella* in pork sold in Mumbai by Acid-pepsin digestion assay and multiplex PCR. Acid-pepsin digestion assay could not able to isolate single larvae from 161 samples similar results were also observed by standardized multiplex PCR. Though none of the sample was found to be positive for *Trichinella* spp. in present study but standardized multiplex PCR assay using standard larvae of *T. spiralis* and *T. britovi* can be useful for differentiation of *T. britovi* and *T. spiralis* larvae in Indian condition. Regular monitoring and surveillance of trichinellosis in pigs and other reservoirs by acid pepsin digestion assay and multiplex PCR is necessary.

Introduction

Trichinellosis, one of the most important food-borne parasitic zoonoses worldwide, is caused by the consumption of raw or under-cooked meat from a wide variety of wild and domestic mammals (Dupouy- Camet, 2000). Pork is consumed across the various pockets of India and it is most important source of infection *to* humans for Trichinellosis. The occurrence of *Trichinella* in domestic animal populations is particularly due to poor management practices which allow pigs to consume food contaminated with *Trichinella* infected meat is the main cause of trichinellosis in pigs (Campbell, 1988). Pigs can only become infected with *Trichinella* by ingesting raw or undercooked meat containing infective larvae. Thus pig is the major source of Trichinellosis in humans.

There are 8 recognized species of *Trichinella* and are grouped under encapsulated and non-encapsulated clad. The different species of *Trichinella* are *Trichinella spiralis* (T-1), *Trichinella native* (T-2), *Trichinella*

britovi(T-3), *Trichinella pseudospiralis* (T-4), *Trichinella murrelli* (T-5), *Trichinella nelsoni*(T-7), *Trichinella papuae* (T-10) and *Trichinella zimbabwensis* (T-11) and four genotypes viz. *Trichinella* T-6, *Trichinella* T-8, *Trichinella* T-9 and *Trichinella* T-12 (Gajadhar *et al.,* 2006 and Gottstein *et al.*, 2009). All these species and genotypes have got zoonotic potential. *Trichinella spiralis* is the most important species because it is most commonly associated with disease in humans and very much adapted to domestic swine with a direct life cycle (Gottstein *et al.*, 2009).

The pigs are important for food security in India. The unhygienic slaughtering of food animals and presence of scavenging pigs is common in India and could be an important risk for occurrence of trichinellosis in humans in India (Singh *et al*., 2013). In India, *Trichinella* has been conclusively isolated from cat, rodents and domestic pigs, (Kalapesi and Rao, 1954; Niphadkar, 1973; and Pethe, 1992; Chetan Kumar, 2011; Jundale, 2015; and Panchal, 2016). In different works various species of *Trichinella* have been isolated from India, mainly *T.spiralis*, *T.britovi* and *T.pseudospiralis* has been reported in country.

Within most parasite genera, distinct morphological and/or biological characters exist amongthe species that permit differentiation and classification. However, other than for *Trichinella pseudospiralis*, the absence of distinguishing morphological characters (Lichtenfels *et al*., 1983) and the overlapping nature of the biological characters (Pozio *et al*., 1992) within the genus *Trichinella* make these traits unsuitable for accurate diagnosis.

The multiplex PCR assay designed by Zarlenga *et al.,* (1999) is the method recommended by the Community Reference Laboratory for *Trichinella* species identification. The PCR allows the comparative analysis of three nucleotide sequences belonging to the internal transcribed spacer 1 and 2 (ITS1 and ITS2) and expansion segment 5 (ESV) of the nuclear ribosomal gene, resulting in the differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*.

In India, very inconsistent literature is available on the burden of Trichinellosis in pigs. Hence, considering these facts and importance of disease the current study was carried out to study exact burden of trichinellosis in pigs of Maharashtra, India.

The aim of the present study was to examine occurrence of *Trichinella* by acid pepsin digestion assay and to standardize multiplex PCR assay to identify two main species of *Trichinella* i.e. *T. spiralis* and *T. britovi*.

Materials and Methods

The present work was carried out at Department of Veterinary Public Health, Bombay Veterinary College, Mumbai. A total of 161 pig diaphragm samples (males-96 and females -65) were collected aseptically from Deonar abattoir, Mumbai. The majority of the pigs slaughtered in the abattoir were of free ranging pigs. The pigs brought to Deonar abattoir from different areas of Maharashtra viz., Dhule, Ratnagiri, Jalgaon, Yerwada, Pune, Nagpur, Palghar, Bhavanipeth and Nanded. The relative information of pigs i.e. place, sex and age etc. was noted down. The pigs were of medium body condition with an average carcass weight of 35 kg (15-55 kg).

Approximately 10-15 g of diaphragm muscle (161), (which is one of the most common predilection sites of *Trichinella* parasite) was collected from pigs.

The diaphragm muscle samples were collected in polyethylene bags and transported to laboratory in chilled condition in an

insulated sample collection box containing ice packs. The diaphragm muscle samples were stored at -18^{0}C till further processing. Prior to process, the samples were thawed in chiller (4-8^{0}C). Then the samples were prepared for the detection of *Trichinella* spp.

All the samples were subjected for identification of *Trichinella* larvae by Acid-pepsin digestion assay as per the protocol of OIE (2012). From each sample, 5 g muscle was weighed and minced then 250 ml of 0.55% Acid (Conc.HCl) and 0.5 g Pepsin (1:10000) was added and transferred into a beaker. Digests were mixed vigorously on a magnetic stir plate at 45° C for 30 min. At the end of 30 min, the digest was allowed to settle and the supernatant was decanted. The sediment was poured through a mesh sieve into separatory funnel and allowed to settle for 30 min, then 10 ml sediment fluid was collected in Petri dish and examined using a stereo microscope at a 10 X magnification.

In the present study, larvae of *T. britovi* and *T. spiralis* were procured from Laboratory of IstitutoSuperiore di Sanita, Department of Infectious, Parasitic and Immuno mediated Diseases, Rome, and used to standardize multiplex PCR assay.

DNA was extracted from larvae as per the procedure described by Guenther *et al.*, (2008) with slight modifications. The micro-centrifuge tube containing larvae was centrifuged at 10,000 rpm for 5 min to allow the larva to settle at the bottom of the tube and excess ethanol was discarded leaving minimum volume. After centrifugation, 2 µl of TRIS–HCl buffer (50mM, pH 7.4–7.6) was added to tube containing *Trichinella* larva in 5 µl distilled water and sealed with a drop of mineral oil. The tube was heated at 90^{0}C for 10 min in hot water bath and cooled to room temperature. Proteinase K (20 mg/ml) 0.4 µl was added to the tube and incubated at 48^{0}C for 3 hrs. At the end of the incubation, the tube was heated at 90^{0}C for 10 min to inactivate the proteinase K. The proteinase K treated larva was used for DNA extraction using DNASure® Tissue Mini Kit (Genetix Biotech Asia, New Delhi) as per the manufacturer's instructions. DNA concentrations were determined spectrophotometrically. Final DNA concentration was adjusted to 200ng by using MiliQ water.

The PCR assay was standardized to amplify the ESV and ITS1 region of nuclear ribosomal gene of the *Trichinella* parasite as per the method described by Zarlenga *et al.*, (1999) with slight modifications. Subsequently a total of 100 randomly selected diaphragm samples (males-60 and females-40) of pig which showed absence of *Trichinella* larvae by HCl-pepsin digestion assay were subjected for DNA extraction by DNASure® Tissue Mini Kit. The isolated DNA from the tissues was used for the multiplex PCR analysis by keeping DNA extracted from standard as a positive control. All the samples showed negative results for *Trichinella* spp.

The PCR was done by using the primers ESV(Forward- 5'-GTT CCA TGT GAA CAG CAG T-3' and reverse-5'-CGA AAA CAT ACG ACA ACT GC-3') and ITS1 (forward-5'-GCT ACA TCC TTT TGA TCT GTT-3' reverse- 5'AGA CAC AAT ATC AAC CAC AGT ACA-3') in order to obtain the best amplification product by optimizing varying the quantity of $MgCl_2$, template DNA concentration, primer concentration, annealing temperature and time. Briefly, the multiplex PCR assay was performed in Master Cycler Gradient Thermocycler (Eppendorf, Germany) having a pre-heated lid. The reaction mixture was performed containing 2.5µl 10x PCR buffer, 1.0µl dNTP Mix (10mM each), 1.0µl $Mgcl_2$ (50mM),

0.5µl each of ESV and ITS1 forward and reverse primers, 1.0µl Taq DNA polymerase (2.5 U/µl), 4µl Template DNA, 1.5µl Glycerol and Nuclease free water to make the total volume 25 µl. PCR assay was performed with an initial denaturation step at 94^0C for 5 min followed by 35 cycles each of denaturation at 94^0C for 1.5 min, annealing at 54^0C for 1 min and extension at 72^0C for 1 min followed by final extension at 72^0C for 5 min. PCR products were kept at -18^0C until further analysis by agarose gel electrophoresis. In each PCR assay, a negative control was also kept. PCR products were separated by 1.5% agarose gel electrophoresis at 95 mA and stained with ethidium bromide.

Results and Discussion

In the present study a total of 161 pig diaphragm samples were analyzed using Acid-pepsin digestion assay but none of the sample was found to be positive for *Trichinella* spp.

The results observed in the present study shows nil occurrences for Trichinellosis in study areas. The previous studies conducted in India suggest nil prevalence of Trichinellosis in pigs (Ramamurthi and Ranganathan, 1968; Pethe and Narsapur, 1992; Gaurat and Gatne, 2005). Studies conducted in Maharashtra reported low prevalence ranges from 0.27% to 0.86% using acid pepsin digestion assay (Jundale, 2015 and Panchal, 2016).

Many studies suggest serological evidence even after negative results by Acid-pepsin digestion assay (Karn, 2007; Konwar *et al.*, 2017). Similarly the directive 77/96/EEC on Pepsin digestion test has a confirmed detection limit of 1-3 larvae/g which may be the reason for non positivity in current study in pigs with low level of infection.

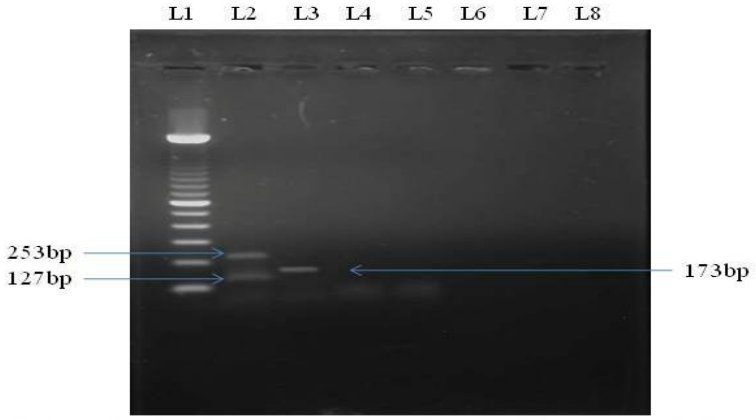

Fig No.1: Electrophoresis pattern of amplified product of samples by multiplex PCR using ESV and ITS1 primers.

Lane 1: 100bp standard DNA ladder
Lane 2: Larval DNA of Standard *T. britovi*
Lane 3: Larval DNA of Standard *T. spiralis*
Lane 4 and 5: Samples
Lane No.7: Negative control

Results from agarose gel electrophoresis of multiplex PCR products using DNA extracted from diaphragm tissue keeping reference strains of *T.britovi* and *T.spiralis* as a positive control are shown in Figure 1. By multiplex PCR assay, none of the sample was found to

be positive for *Trichinella*. Standardized PCR results indicate indicate unique and simple banding patterns for each of the genotypes. Amplified products of *T. britovi* showed genotype fragment size of 127 and 253 bp for ESV and ITS1 primers, respectively. Whereas, *T. spiralis* showed only one genotype fragment size of 173 bp for ESV. This indicates that standardized cycling conditions in this multiplex PCR can be useful for differentiation of *T. britovi* and *T. spiralis* larvae in Indian condition. The standardized multiplex PCR assay was to be used for identifying all genotypes and species of *Trichinella* larvae, if the larvae would have been isolated from tissues by Acid-pepsin digestion assay.

Various workers used Multiplex PCR assay for differentiating species of *Trichinella* in different geographical conditions and for different strains (Kapel *et al.,* 2001; Pozio *et al*., 2004; Hurnıkova *et al*., 2005; De Bruyne *et al.,* 2005 Merialdi *et al*., 2011 andKirjusina *et al.,* 2015). Among the EVS and ITS1 primers, ESV is the only nucleotide sequence present in all species of *Trichinella* but it is highly variable in size and nucleotide sequence for each *Trichinella* spp. However ITS1 nucleotide sequence is present only in *T. britovi*. Thus this method can be useful to differentiate between *T.spiralis* and *T.britovi* which are reported in India. Along with this, standardized PCR can be used to differentiate all species of *Trichinella* due to its unique banding pattern for ESV primers in each species. Thus this method is simple, specific and cost effective for diagnosis of *Trichinella* spp.

The current study demonstrated non detectable occurrence of Trichinellosis in domestic pigs by Acid –pepsin digestion assay and multiplex PCR assay but it is necessary to study epidemiological situation of parasitic diseases. Regular monitoring and surveillance by acid pepsin digestion assay and multiplex PCR in synanthropic animals like rodents, other domestic animals and wildlife is essential to have a complete scientific data on prevalence of *Trichinellosis* in India.

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Conflict of interest: The authors declare that they have no any conflict of interest.

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