



Copro-prevalence of *Cryptosporidium* in pigs of selected districts in West coast of India: A preliminary study

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ABSTRACT

In India, *Cryptosporidium* spp. have been detected and characterised from humans and domestic animals, mostly ruminants. Although pigs can act as an important reservoir of *Cryptosporidium* infection to humans, comprehensive studies have not been conducted in pig population in India. Hence, present study attempt to elucidate the prevalence and diversity of *Cryptosporidium* infection in pigs in the selected districts of three states situated in the West coast of India. The pig faecal samples (n=221) were subjected to coprological examination using modified Ziehl Neelsen (mZN) staining method, followed by polymerase chain reaction to detect *Cryptosporidium* spp. Using mZN staining method, 1.35% (95% CI, 0.46%–3.91%) of the samples were found positive for *Cryptosporidium* oocyst. However, the DNA from *Cryptosporidium* positive faecal samples could not be amplified using polymerase chain reaction probably because of the low sensitivity of the PCR and or low oocyst number in the faecal sample. *Cryptosporidium* spp. of pig origin have zoonotic potential and a certain proportion of pigs infected with *Cryptosporidium* may be apparently healthy. Therefore, the pig farmers need to be made aware of hygienic practices while handling pigs and pig manure and general public must be made sensitised about the good agricultural practices and standard food hygiene practices to prevent foodborne *Cryptosporidium* infection.

Keywords: *Cryptosporidium*, India, Pigs, Prevalence, West coast

Cryptosporidium is an intestinal protozoan parasite infecting mammals, reptiles, amphibians, fish, birds and man (Certad *et al.* 2019). Cryptosporidia have public health significance as some of the *Cryptosporidium* species are transmissible between animals and humans. At least 38 species and same number of genotypes of *Cryptosporidium* exist (Feng *et al.* 2018). Although humans can get infected with several species of *Cryptosporidium*, the two most common species reported worldwide in humans are *C. hominis* and *C. parvum* (Innes *et al.* 2020). In humans, *Cryptosporidium* is responsible for severe diarrhoea in immuno-compromised individuals (Ryan *et al.* 2014). It is estimated that annually between 58,000–146,000 deaths are reported in children under the age of two years due to *Cryptosporidium* infection in India (Sarkar *et al.* 2014). Faeco-oral transmission through food and water contaminated with oocysts or direct contact with

infected animals are the major route of transmission of *Cryptosporidium* (Limaheluw *et al.* 2019). In India, several studies to identify the prevalence of *Cryptosporidium* have been conducted in livestock mostly in ruminants with very few investigations in pigs (Kumar *et al.* 2004, Mallinath *et al.* 2009, Rajendran *et al.* 2011, Murya *et al.* 2013, Joute *et al.* 2016, Dixit *et al.* 2019, Patra *et al.* 2019). Pigs can harbour several species of *Cryptosporidium*, viz. *C. suis*, *C. scrofarum*, *C. muris*, *C. tyzzeri* and *C. parvum*. However, the major *Cryptosporidium* species identified in pigs worldwide are *C. suis* and *C. scrofarum* (Ryan *et al.* 2014). The host adapted *Cryptosporidium* species of pigs, viz. *C. suis* and *C. scrofarum*, have also been shown to infect humans that indicate the zoonotic potential of these two species and warrants comprehensive studies in pigs as well (Xiao *et al.* 2002, Leoni *et al.* 2006, Kvac *et al.* 2009, Bodager *et al.* 2015).

In India, the prevalence of *Cryptosporidium* spp. and *Giardia* spp. in livestock (other than pigs) from coastal region has been reported previously (Daniels *et al.* 2015). Further, no comprehensive studies have been conducted to identify the prevalence of *Cryptosporidium* in pigs reared in coastal districts of India. Therefore, the present study was undertaken with the objective to identify the prevalence and species diversity of *Cryptosporidium* in pigs reared

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in selected coastal districts in three West coastal states of India, viz. Goa, Karnataka and Maharashtra.

MATERIALS AND METHODS

Study location: The study was undertaken in the five districts in the three states adjoining the West coast line of India, viz. North Goa and South Goa district (Goa state), Palgarh and Sindhudurg district (Maharashtra state) and Dakshina Kannada district (Karnataka) (Table 1). The districts were chosen based on logistics and convenience of sampling. The common pig breeds reared in this region were Agonda Goan, Large White Yorkshire, and cross bred between the native pigs with Large White Yorkshire or Duroc breeds. The pigs were housed either in confinement housing or loose housing system. The holding size of pigs ranged from one pig to few hundred pigs.

Table 1. Details of pig faecal sample collected from different locations

State	District	Age group (months)				Total
		0-3	3-6	6-12	> 12	
Goa	North Goa	25	28	34	29	116
	South Goa	3	5	25	15	48
Maharashtra	Palgarh	4	4	0	6	14
	Sindhudurg	3	0	0	0	3
Karnataka	Dakshina	7	13	12	8	40
	Kannada					
Total		42	50	71	58	221

Sample collection: A convenient sampling was undertaken between January 2016 and April 2019 and a total of 221 pig faecal samples were collected. The number of pigs sampled from loose/scavenging and confinement housing were 58 and 163, respectively. The approximate age of the pigs were obtained from the farmers that ranged from two weeks to more than 3 years. Freshly voided pig faeces (approximately 5-10 g) from the ground were collected in sterile sample container (Himedia®, Mumbai) and transported to the laboratory in chilled condition. At the time of collection the consistency of faeces was recorded as formed (non-diarrhoeic) (n=218) and watery (diarrhoeic) (n=3). When immediate examination was not possible, the faecal samples were stored at 2-4°C. From each animal the sample was collected only once. In addition to *Cryptosporidium* oocyst detection, the faecal samples were also examined for the presence other common intestinal parasites.

Modified Ziehl Neelsen (MZN) staining: Faecal samples were stained with modified Ziehl Neelsen (mZN) staining technique for the detection of *Cryptosporidium* oocysts. Briefly, suspension of faeces was prepared by mixing a peanut sized faecal sample with distilled water. The resulting suspension was spread over a glass slide to obtain a thin fecal smear. The smear was air dried and fixed with absolute methanol for 5 min. The slide was flooded with strong carbol fuchsin (Himedia, Mumbai) for 15 min and rinsed with tap water. Then the smear was decolorised

using acid fast decoloriser (Himedia, Mumbai) for 10 s followed by rinsing with tap water. The smear was counter stained with 0.4% aqueous malachite green for 1 min. Finally the slide was rinsed with tap water, air dried and examined under oil immersion objective. For each slide, a total of 20 fields were examined and the number of oocysts were counted. If no oocysts were detected in the 20 fields then the whole slide was examined.

Molecular identification of *Cryptosporidium*: DNA extraction, The DNA was extracted from pig fecal samples which were found positive for *Cryptosporidium* by mZN staining. In addition DNA was also extracted from another 50 fecal samples which were found negative by MZN staining. Genomic DNA was extracted from 200 µg feces using either QIAamp® DNA stool mini kit or QIAamp® fast DNA stool mini kit (Qiagen, Germany) as per the manufacturer's instruction except for the final DNA elution, wherein DNA was eluted in a volume of 65 µl. A bovine dung sample previously confirmed positive for *Cryptosporidium andersoni* by polymerase chain reaction (kindly provided by Dr. Venu R, College of Veterinary Science, Tirupati) was used as reference faecal sample to assess the efficacy of DNA extraction procedure and as a positive control in polymerase chain reaction. The DNA concentration and purity was measured using Nanodrop Spectrophotometer (Thermoscientific).

Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP): For species identification of the *Cryptosporidium*, the PCR-RFLP protocol was standardised as per the method described by European Union Reference Laboratory for Parasites, Istituto Superiore di Sanità, Rome, Italy with necessary modifications (EURLP 2015). Briefly, the gene encoding for *Cryptosporidium* oocyst wall protein, (COWP) was amplified using Cry9 (5'-GGACTGAAATACAGGCATTATCTTG-3') and Cry15 (5'-GTAGATAATGGAAGAGATTGTG-3') internal primers. The reaction mixture for polymerase chain reaction (PCR) consisted of 2×Taq PCR master mix (Qiagen, Germany), 10 pmol each of forward and reverse primers, 0.125 µl bovine serum albumin (20 mg/ml) and 5 µl faecal DNA template and nuclease free water to make the volume to 25 µl. The thermal cycling conditions were initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 49°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 7 min. The resulting PCR amplicon was electrophoresed in 1.5% agarose gel to visualise the expected 553 bp amplicon.

Later, the positive PCR amplicon was subjected to restriction enzymes digestion using *RsaI* enzyme. The reaction mixture for restriction enzyme digestion consisted of 2 µl of 10× RE buffer, 5 units of *RsaI* restriction enzyme, 10 µl of PCR product and nuclease free water to make the volume to 20 µl. The mixture was incubated at 37°C for 4 h followed by gel electrophoresis with 3% agarose and visualised under UV light (EURLP 2015). The DNA isolated from *Cryptosporidium andersoni* reference faecal

sample was used as positive control and nuclease free water as negative control for standardisation of PCR and RFLP protocols.

RESULTS AND DISCUSSION

Copro-prevalence of Cryptosporidium spp. in pig faeces: Out of 221 pig fecal samples examined, 3 fecal samples (one from south Goa district and two from North Goa district) were found positive for *Cryptosporidium* oocyst by mZN staining technique (Fig. 1). Thus, the prevalence of *Cryptosporidium* oocysts was 1.35% (95% CI, 0.46%-3.91%) by mZN staining technique. The number of oocysts in all the three faecal samples was ≤ 5 oocysts per slide. The consistency of all the three faecal samples was formed and non-diarrhoeic. Among the three positive pigs, two pigs were reared in loose housing system and one pig in confinement system. The three pigs positive for *Cryptosporidium* were one each from 3-6 months, 6-12 months and > 12 months age group. None of the pigs aged less than 3 months were positive for *Cryptosporidium*. The fecal samples of pigs collected from Karnataka and Maharashtra states were negative for *Cryptosporidium* oocyst.

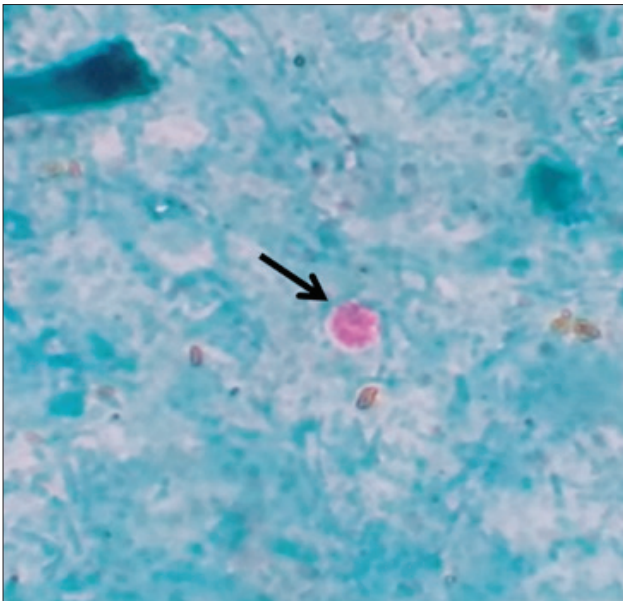


Fig. 1. *Cryptosporidium* oocyst in a MZN stained pig faeces (1000 \times).

The prevalence of *Cryptosporidium* spp. in pigs recorded in the present study was much lower compared to the studies of Maurya and co-workers whose study revealed the prevalence of 19.1% in the state of Uttar Pradesh (Maurya *et al.* 2013). In a recent study by Patra *et al.* 2019 in the six North Eastern states of India the prevalence of *Cryptosporidium* in pig faeces was found to be 10.66% which was also higher than the prevalence recorded in the present study (Patra *et al.* 2019). Both the aforementioned studies were conducted in mainland territories. Upon literature search there was one survey conducted in Pondicherry located in the East coast of India where in

only five pig feces were examined for *Cryptosporidium* with negative result (Kumar *et al.* 2004). It was evident that no extensive studies involving large sample size was available from coastal region to compare the result of the present study.

In the study of Maurya *et al.* (2013) and Sanford (1987), the prevalence of *Cryptosporidium* was high in non-diarrhoeic piglets than those suffering from diarrhoea. Although in the present study the number of diarrhoeic pig feces examined was less to compare with the non-diarrhoeic group, the *Cryptosporidium* was found only in non-diarrhoeic pig feces that were apparently healthy. Present findings corroborates with that of Villacorta *et al.* (1991) who recorded *Cryptosporidium* infection from apparently healthy piglets (Villacorta *et al.* 1991). The absence of *Cryptosporidium* from fecal samples of pigs from Karnataka and Maharashtra may be because of less number of fecal samples examined from these two states. Had it been a higher sample size there would have been a greater probability of detecting *Cryptosporidium* from the pigs of aforementioned states.

Molecular identification of Cryptosporidium spp : Using PCR, a 553 bp fragment of COWP gene was successfully amplified from DNA of *C. andersoni* reference positive control stool sample (Fig. 2). Further, the positive PCR amplicon was subjected to RFLP using the enzyme *RsaI* and the expected number of fragments were obtained (Fig. 3). However, the DNA of three fecal samples positive by mZN staining and 50 faecal samples negative by mZN staining failed to amplify in PCR.

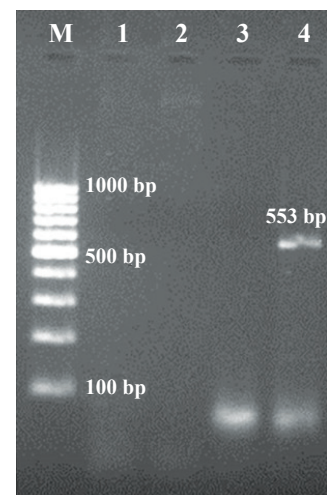


Fig. 2. Standardization of PCR for detection of *Cryptosporidium* spp. (Lane M: 100 bp DNA ladder, Lane 1 and 2: Pig fecal DNA positive by MZN staining, Lane 3: Negative control, Lane 4: Positive control).

The lack of amplification of mZN positive faecal specimen by PCR can happen because of several reasons, viz. faulty DNA extraction, not using enough quantity of template DNA, DNA degradation, inhibition of PCR due to inhibitors in stool samples, etc. However, it is possible that the aforementioned factors may not be responsible for the lack of amplification in present study. The most

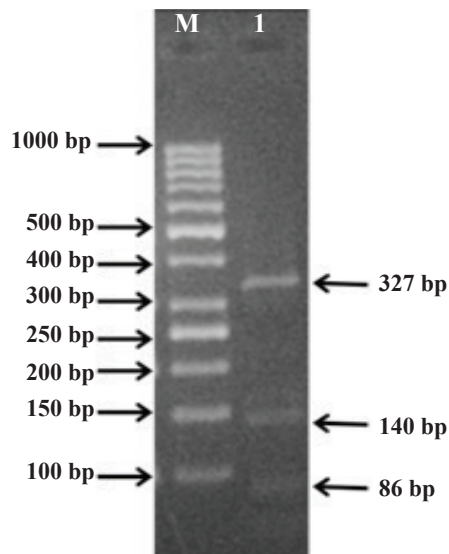


Fig. 3. Standardization of RFLP using *Rsa* I restriction enzyme for the detection of *Cryptosporidium* spp. (Lane M: 50 bp DNA ladder, Lane 1: Positive control).

plausible reasons for lack of amplification could be the lower sensitivity of PCR protocol targeting the COWP gene and less number oocysts present in the faecal samples. Previously, it has been shown that nested PCR amplification of COWP gene using two primer pairs, viz. external and internal primer pairs was more sensitive than using internal primer pair alone (Pedraza-Diaz *et al.* 2001). In the present study only the internal primer pair in PCR amplification was used. Further, the PCR protocol targeting 18S rRNA gene is likely to be more sensitive than COWP gene used in the present study because of the higher copy number of the former (Pedraza-Diaz *et al.* 2001). It is also interesting to note that, higher the number of oocysts present in the fecal sample, better the chance of amplification in PCR (McLauchlin *et al.* 1999). Considering the lower number of oocysts (≤ 5 oocysts per slide) detected in the faecal sample, the lack of amplification was not unusual.

The previous studies have confirmed the existence of *Cryptosporidium* in livestock as well as humans in mainland areas of India. However, studies on *Cryptosporidium* in livestock in coastal regions are minimal (Kumar *et al.* 2004, Daniels *et al.* 2015). To the best of our knowledge and based on literature survey of published research, this is the first study to identify the prevalence of *Cryptosporidium* in the pigs reared in the West coastal region of India. The attempt to determine the species of *Cryptosporidium* using polymerase chain reaction was unsuccessful.

In conclusion, although in the present study the prevalence of *Cryptosporidium* in pigs was found to be very low, considering the zoonotic potential *Cryptosporidium* species of pig origin, pig farmers must be made aware of the hygienic practices and personal protection measures to be followed while handling pigs and pig manure (Thathaisong *et al.* 2020). The general public must be aware of good agricultural practices and follow standard food hygiene measures to prevent food-borne cryptosporidiosis.

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