VETERINARSKI ARHIV 91 (3), 269-276, 2021 DOI: 10.24099/vet.arhiv.0674

Genetic diversity within the 18S rRNA and *actin* locus of *Cryptosporidium scrofarum* (Apicomplexa: Cryptosporidiidae) infecting domestic pigs (*Sus scrofa domesticus*) of India

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SHARMA, D., N. K. SINGH, H. SINGH, S. S. RATH, D. P. BLAKE: Genetic diversity within the 18S rRNA and *actin* locus of *Cryptosporidium scrofarum* (Apicomplexa: Cryptosporidiidae) infecting domestic pigs (*Sus scrofa domesticus*) of India. Vet. arhiv 91, 269-276, 2021.

ABSTRACT

The genetic diversity was studied of *Cryptosporidium scrofarum* (syn *Cryptosporidium* pig genotype II) of domestic pigs (*Sus scrofa domesticus*) from Punjab, India. Nested PCR amplification targeting the 18S rRNA and *actin* gene loci from *Cryptosporidium* positive samples was carried out, and the amplicons were sequenced. Phylogenetic comparison of a partial 18S rRNA gene revealed that they were genetically most similar to *C. scrofarum* isolated from other parts of the world. However, comparison of sequences representing a fragment of the genomic *actin* locus identified a new genotype conserved within the isolates sampled from India but distinct from other published sequences, suggesting the presence of a different Indian genotype.

Key words: *actin*; *Cryptosporidium scrofarum;* genetic diversity; pig; phylogeny; 18S rRNA

Introduction

Protozoa of the genus *Cryptosporidium* are apicomplexan parasites which inhabit the digestive and respiratory systems of birds, fish, reptiles, and mammals, including humans, with worldwide distribution (XIAO, 2010). Extensive genetic variation has been recorded within the genus *Cryptosporidium,* with 27 species and more than 70 genotypes recognized to infect humans and/or animals (LIN et al., 2015). From pigs, six *Cryptosporidium* species have been isolated globally, *viz*., *C. suis*, *C. parvum*, *C. muris*, *C. andersoni*, *C. scrofarum* (formerly named *Cryptosporidium* sp. pig genotype II) and *C. tyzzeri* (formerly named *Cryptosporidium* sp. mouse genotype I) (KVAC

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ISSN 0372-5480
Printed in Croatia

et al., 2013; YUI et al., 2014). Reports have indicated zoonotic potential for both *C. suis* and *C. scrofarum*, where humans were found to have been infected with these pig-derived *Cryptosporidium* species (XIAO et al., 2002; KVAC et al., 2009) Cryptosporidiosis in pigs occurs primarily through transmission by the faeco-oral route, commonly resulting in diarrhoea and weight loss, however, in neonatal and immunodeficient pigs, mortality has also been observed (BOUZID et al., 2013).

The occurrence of *Cryptosporidium* infections in domestic pigs (*Sus scrofa domesticus*) has been reported worldwide (RYAN et al., 2003; SUAREZ-LUENGAS et al., 2007; JOHNSON et al., 2008; CHEN et al., 2011; YIN et al., 2011; DA SILVA FIUZA et al., 2011; BUDU-AMOAKO et al., 2012; NEMEJC et al., 2013). Pigs are an important reservoir of *Cryptosporidium,* which thus makes it imperative to understand its prevalence in swine for the prevention and control of cryptosporidiosis in both animal and human populations. Differences have been reported in the population structure and molecular characteristics of *Cryptosporidium* species/genotypes in pigs between and within countries (ZHANG et al., 2013), encouraging detailed regional screening. In India, however, no published reports are available on the occurrence, molecular characterization and phylogenetic analysis of pig-derived *Cryptosporidium* isolates. Therefore, the present study aimed to characterize *Cryptosporidium* sp. and identify the genetic diversity at the 18S rRNA and *actin* gene loci.

Materials and methods

Collection and coproscopic examination. Faecal samples (n = 839) were collected from apparently healthy pigs from 36 organised or backyard pig farms in Punjab State, India. The faecal samples were subjected to routine coprological studies, and samples (n = 43) positive for coccidian oocysts were transported to the Royal Veterinary College for molecular characterisation studies. These were screened for concurrent *Cryptosporidium* infection by a modified Ziehl Neelsen staining technique (BHAT et al., 2014) and nested polymerase chain reaction at two gene loci, viz. the 18S rRNA and *actin* gene, as described below.

Subheading genomic DNA extraction. The positive samples were subjected to genomic DNA extraction, as described previously for coccidian parasites (KUMAR et al., 2014). Briefly, an aliquot of ~200 mg sample was first homogenised using a Bead Beater at 30,000× oscillations/min for 30 sec after adding 0.4-0.6 mm glass beads (Sigma-Aldrich, St Louis, USA) to 0.5 volume of the faecal pellet. Total genomic DNA was subsequently isolated using a QIAamp DNA Stool mini kit (Qiagen, Germany), as per the manufacturer's protocol with some modifications. Each homogenized faecal sample was mixed with 1.4 mL ASL buffer in a 2.0 mL microcentrifuge tube. The suspension was then heated for 5 min at 70 °C and processed as per the kit protocol. The DNA was eluted twice in 100 µL Tris EDTA (TE) buffer, quantified, and the purity was checked using a Nanodrop 2000/200C spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20 °C until use.

18S rRNA gene amplification and sequencing. A fragment of the *Cryptosporidium* 18S rRNA gene was amplified as described by XIAO et al. (2001) using the primers

Crypto F1 (5′-TTCTAGAGCTAATACATGCG-3′)
and
Crypto R1 5′-CCCATTTCCTTCGAAACAGGA-3′),
followed by
Crypto iF2 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′)
and
Crypto iR2
(5′-AAGGAGTAAGGAACAACCTCCA-3′).

Each reaction was performed in a final volume of 25 µL in a thermal cycler (Applied Biosystems™ SimpliAmp™). In the primary assay 2.5 µL (~2-20 ng/µL) genomic DNA was used as template, together with 400 nM forward and reverse primers, and 12.5 µL of 2× MyTaq™ Mix (Bioline, Taunton, USA), made up to 25 µL with nuclease free water (Thermo-Fisher Scientific, Hemel Hempstead, UK). The PCR amplification was initiated at 94 ºC for 3 min followed by 40 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 1 min and a final elongation step at 72 ºC for 7 min. In the nested assay, 1 µL of the primary PCR amplicon was used as a template, with the same reaction mixture and cycling conditions as the primary assay, other

than substitution of the nested primers. Total genomic DNA from Cryptosporidium sp. hedgehog genotype (SANGSTER et al., 2015) and nuclease free water served as positive and negative controls, respectively. PCR amplicons were viewed on 1.0% (w/v) agarose gel stained with 0.01% SafeView™ Nucleic Acid Stain (Novel Biological Solutions, Huntingdon, UK). Amplicons of the correct size were purified using a QIAquick® PCR Purification Kit (Qiagen, Germany) as per the manufacturer's instructions, and sequenced using the internal nested primers. The positive amplicons were subjected to two-directional sequencing, with internal primers used for nested PCR assay using the ABI Ready Reaction Mix (BigDye® Terminator v3.1 chemistry; Applied Biosystems, Foster City, USA), followed by direct automated sequencing (GATC Biotech, Cologne, Germany).

Partial actin gene amplification and sequencing. A semi-nested PCR was employed to amplify a fragment of the genomic *actin* locus using the primers ScrofActinFA (5'-TGTAGGTGACGAGGCTCAATCCAA-3') and ScrofActinRA (5'-ATCGATTGGAAAGTGGTCTCGCCA-3'), followed by ScrofActinFA and ScrofActinRB (5'-TTCTGGGCACCTAAATCTCTCGCT-3').

Each reaction was performed in a final volume of 25 µL in a thermal cycler (Applied Biosystems™ SimpliAmp™). In the primary assay, 2.5 µL (~2-20 ng/µL) genomic DNA was used as the template, together with 400 nM forward and reverse primers and 12.5 µL of 2× MyTaq™ Mix (Bioline, Taunton, USA), made up to 25 µL with nuclease free water (Thermo-Fisher Scientific, Hemel Hempstead, UK). The PCR amplification was initiated at 94 ºC for 5 min followed by 35 cycles of 94 ºC for 45 sec, 57 ºC for 45 sec, 72 ºC for 1 min, and a final elongation step at 72 ºC for 10 min. In the nested assay, 1 µL of the primary PCR amplicon was used as a template with the same reaction mixture, and the cycling conditions were the same as the primary assay, other than the substitution of the nested primers, as described by KVAC et al. (2013).

Sequence analysis. Sequences were assembled and curated using CLC Main Workbench version 6, with consensus sequences annotated on the basis of BLASTn similarity using the GenBank non-redundant dataset. Related sequences from other *Cryptosporidium* isolates were identified, downloaded and aligned using MUSCLE, prior to phylogenetic analysis using MEGA 6.0. Molecular phylogenetic analysis was conducted using the Maximum Likelihood method in Mega 6.0 software with the Tamura 3-parameter model, based upon the optimal Akaike Information Criterion. The rate variation was modelled with a gamma distribution and invariant sites, supported by 1000 bootstrap replication. Neighbor Joining and Maximum Parsimony were run in parallel for comparison of phylogenetic stability. Sequences representing a fragment of the genomic *actin* locus were processed in the same way, using the General Time Reversal model with invariant sites.

Results

The nested polymerase chain reaction (PCR) analysis of the DNA extracted from the *Eimeria* positive samples originating from the Fazilka and Ludhiana regions of Punjab, India, resulted in amplification of a partial 18S rRNA fragment of ~821 bp. The *Cryptosporidium* oocysts were also identified by a modified Ziehl Neelsen staining technique (Fig. 1).

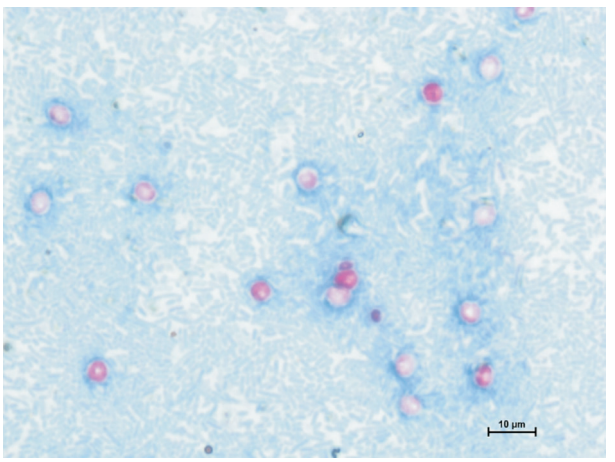


Fig. 1. *Cryptosporidium* oocysts by modified Zeihl Neelsen Staining, ×100.

The sequencing and BLAST analysis of the amplicons revealed that they were most similar to *C. scrofarum* (syn *Cryptosporidium* genotype II).

Phylogenetic comparison of these 18S rRNA gene sequences (accession numbers MG576146-7) from the Fazilka and Ludhiana regions of Punjab, India, with other published *C. scrofarum* 18S rRNA sequences, and a selection of other reference sequences, revealed a single *C. scrofarum* lineage (Fig. 2a).

Comparable topologies were determined using ML, NJ and MP methods. Phylogenetic comparison of 619 bp fragments of the *C. scrofarum* genomic *actin* locus from Fazilka and Ludhiana samples (LT976831-2), with reference sequences from Japan and the Czech Republic (AB852580 and JX424841), identified two shared synonymous substitutions in both Indian sequences (Supplementary Fig. 1). An additional, unannotated sequence from Norway was also compared (EF012374), revealing two different synonymous and one non- synonymous substitutions (Supplementary figures Figs. 1 and 2). Phylogenetic comparison of the genomic *actin* sequences revealed a genetic distance between the Indian and the other *C. scrofarum* sequences (Fig. 2b).

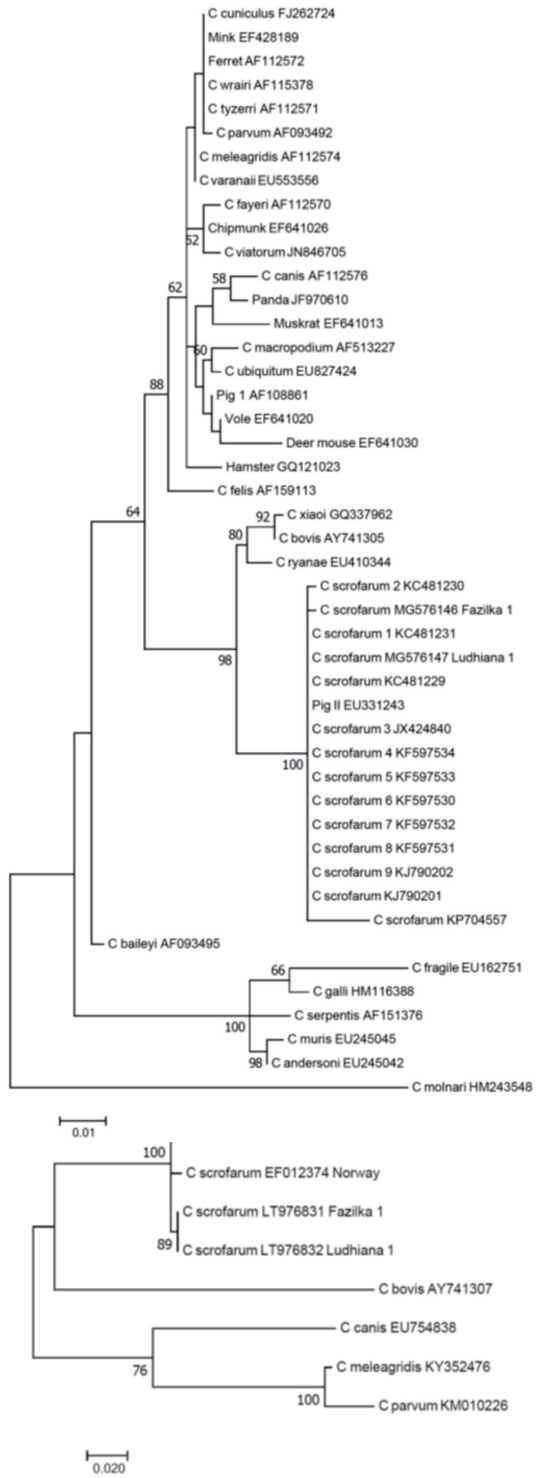
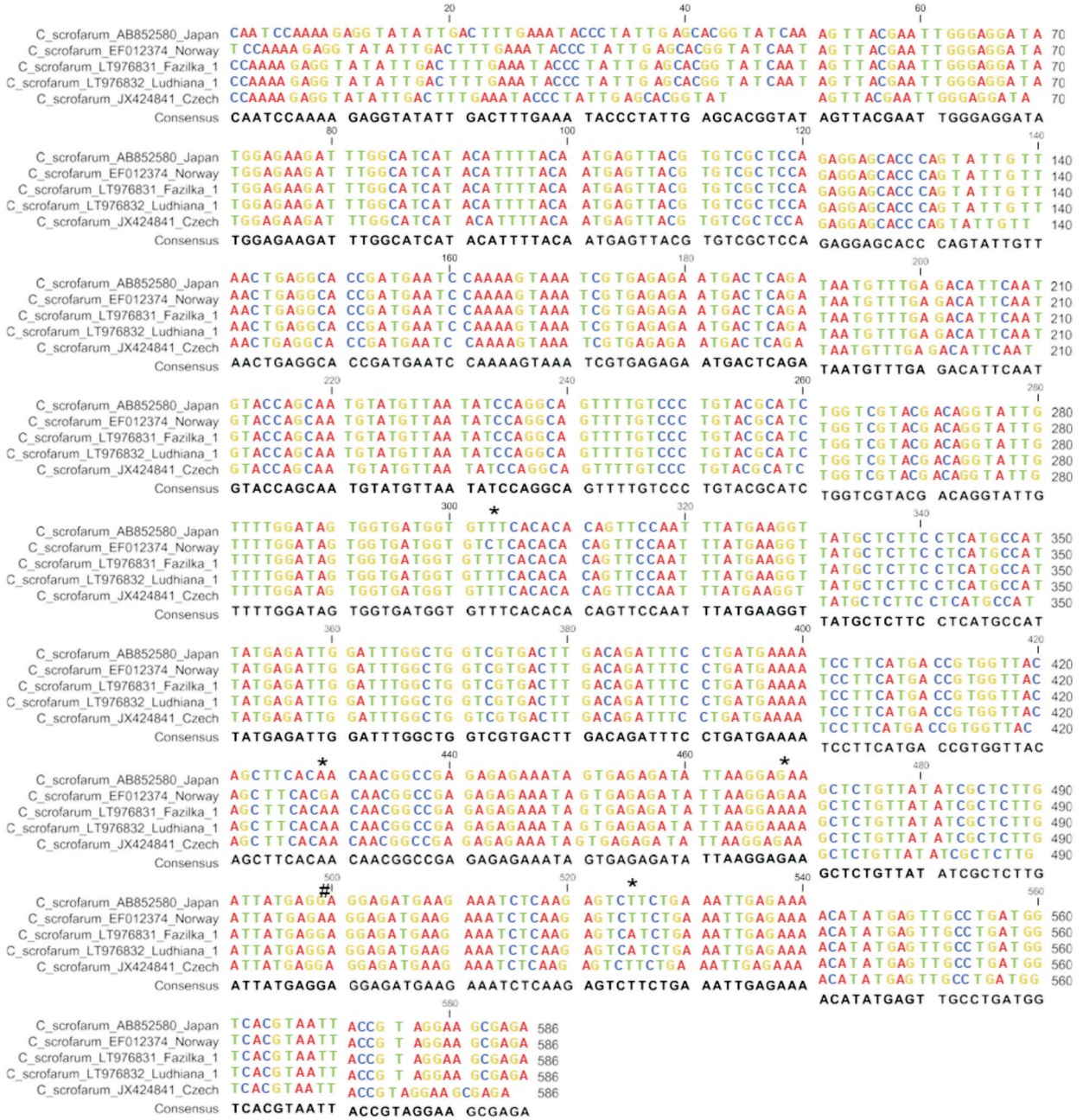


Fig. 2. Maximum Likelihood phylogenies based on partial (a) 18S rDNA and (b) *actin* gene sequences of *Cryptosporidium* sp. Assemblies of 705 and 586 bp were used, respectively. Neighbor Joining and Maximum Parsimony methods provided comparable topologies.

Discussion

RYAN et al. (2003) in their study on molecular characterization of *Cryptosporidium* from pigs employing 18S rRNA, identified two distinct genotypes of *Cryptosporidium* sp., namely genotype I (*C. suis*) which was previously known from pigs, and a novel pig genotype (pig genotype II). They suggested that this novel genotype warranted species status. Later, KVAC et al. (2013) proposed the species name *C. scrofarum* to reflect its prevalence in adult pigs worldwide. In the present study, *C. scrofarum* was identified for the first time in pigs in Punjab state, and to the best of our knowledge there are no previous reports from India. Cryptosporidiosis in pigs has occasionally been shown to result in clinical signs. In humans, the two pig-adapted *Cryptosporidium* species, *viz*. *C. suis* and *C. scrofarum,* are potentially zoonotic (XIAO et al., 2002; CAMA et al., 2003; LEONI et al., 2006; KVAC et al., 2009).

Cryptosporidium *scrofarum* has been also detected in an immuno-competent person, in cattle without pigs nearby, and from a potential human source (DA SILVA FIUZA et al., 2011).



Supplementary Fig. 1. Alignment of published and new *C. scrofarum* partial genomic *actin* locus sequences. * = Synonymous substitution, # = Non-synonymous substitution.

Supplementary Fig. 2. Alignment of published and new *C. scrofarum* predicted *actin* amino acid sequences. # = Non-synonymous substitution.

Other Cryptosporidium species isolated from pigs, namely; C. suis, C. muris and C. parvum, have also been associated with zoonotic infections (YIN et al., 2011). Thus, pigs principally free roaming, pose a significant public health risk because they act as an important reservoir of Cryptosporidium sp. and can facilitate zoonotic infections via faecal contamination of water sources and the environment.

Phylogenetic comparison of a partial 18S rRNA gene fragment identified a distinct monophyletic grouping for *C. scrofarum*, most closely related to *C. bovis*, *C. xiaoi* and *C. ryanae*, but distinct from *C. parvum* and other related species in line with KVAC et al. (2013b) propose the species name *Cryptosporidium scrofarum* n. sp. to reflect its prevalence in adult pigs worldwide. Oocysts of *C. scrofarum* are morphologically indistinguishable from C parvum, measuring 4.81-5.96 \u03bcm (mean = 5.16). XIAO et al. (1999) has previously associated this difference with the biology of the two *Cryptosporidium* groups, including different predilection sites. Additional analysis using a fragment of the genomic *actin* locus revealed distinct differences between the Indian and all other published sequences, suggesting the presence of a different Indian genotype. Additional samples need to be sequenced for validation. Such genetic markers offer value in future studies of the occurrence, genetics and diversity of *C. scrofarum*.

In conclusion, we report for the first time *Cryptosporidium scrofarum* (syn *Cryptosporidium* genotype II) from pigs reared in India. Because of the asymptomatic infections caused by *C. scrofarum* and *C. suis* in pigs, and the close contact of domestic pigs with humans and human water sources, these parasites pose an invisible threat to human health. Further, pigs can act as reservoirs of cryptosporidiosis for onward transmission to immune-compromised individuals. Intra-species comparison of a partial *actin* gene fragment indicated polymorphism between samples sequenced in this and other published studies. Thus, further molecular epidemiological surveillance is required to assess transmission dynamics in pigs, and to formulate effective control strategies.

Acknowledgments

The authors are grateful to the Dean of Post-Graduate Studies, Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab, India, for providing the facilities to carry out the research work. Thanks are also due to the farmers who generously gave their time and hospitality. Sincere thanks to the Commonwealth Scholarship Commission for granting funds to the senior author through a Split Site PhD Scholarship (ID: INCN-2016-68) to carry out part of the work at the Royal Veterinary College, United Kingdom.

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Received: 29 March 2019
Accepted: 12 March 2021

SHARMA, D., N. K. SINGH, H. SINGH, S. S. RATH, D. P. BLAKE: Genetička raznolikost unutar 18S rRNA i aktin-lokusa *Cryptosporidium scrofarum* (Apicomplexa: Cryptosporidiidae) pri invaziji domaćih svinja (*Sus scrofa domesticus*) u Indiji. Vet. arhiv 91, 269-276, 2021.

SAŽETAK

U ovom je radu istraživana genetička raznolikost *Cryptosporidium scrofarum* (syn Cryptosporidium pig genotype II) domaćih svinja (*Sus scrofa domesticus*) iz regije Punjab, Indija. Provedeno je umnožavanje 18S rRNA i lokusa *aktin*-gena pomoću ugniježđene PCR metode iz uzoraka pozitivnih na *Cryptosporidium* te su sekvencirani amplikoni. Filogenetska usporedba parcijalnog 18S rRNA gena pokazala je da su uzorci genetski najsličniji *C. scrofarum* izoliranom u drugim dijelovima svijeta. Također, usporedba sekvencija dijela lokusa genomskog aktina otkrila je novi genotip očuvan unutar izolata uzorkovanih u Indiji, ali različitih od drugih objavljenih sekvencija, upućujući na postojanje zasebnog indijskog genotipa.

Ključne riječi: *aktin; Cryptosporidium scrofarum*; genetička raznolikost; svinja; filogenija; 18S rRNA