**Identification of pig-specific *Cryptosporidium* species in mixed infections using Illumina sequencing technology**

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Short Communication

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**Abstract**

Nowadays molecular methods are widely used in epidemiological studies of *Cryptosporidium* infections in humans and animals. However to gain better understanding of parasite species   
or genotypes, especially when mixed infections are noticed, highly sensitive tools with adequate resolution power need to be employed. In this article, we report an application of the next generation sequencing method (NGS) for detection and characterisation   
of *Cryptosporidium* species concurrently present in pig faeces. A mixture of *Cryptosporidium* DNA obtained from two faecal samples was amplified at the 18 SSU rRNA gene locus and the resulting amplicons were subsequently used for MiSeq sequencing. Although initial molecular analyses indicated the presence of *Cryptosporidium scrofarum*, *Cryptosporidium suis,* and an unidentified species, deep sequencing only confirmed the presence of pig-specific *Cryptosporidium*. In addition, NGS showed its usefulness in identification   
of *Cryptosporidium* species concurrently present in faecal samples characterised by low abundance of parasite DNA.

Keywords: *Cryptosporidium,* Mixed infections, NGS, Identification

1. Introduction

Molecular investigation of *Cryptosporidium* invasions was first conducted in the 1990s   
by Laxer et al. (1991) who used a PCR method for the detection of parasite DNA in human and cattle faecal samples. Since that time better recognition of the *Cryptosporidium* genome has enabled rapid development of several detection methods (Vesey et al., 1995; Homan et al., 1999; Baeumner et al., 2001; Glaberman et al., 2001; Higgins et al., 2001; Sulaiman et al., 2001) and identification methods for parasite species (Spano et al., 1997; Homan et al., 1999; Xiao et al., 1999; Sulaiman et al., 2000) or their subgenotypes (Glaberman et al., 2002; Sulaiman et al., 2005). Molecular methods are also irreplaceable in determining the taxonomic affiliation of the identified parasites (Fayer and Santín, 2009). Nowadays, they are widely used in epidemiological studies on *Cryptosporidium* infections in humans (Iqbal et al., 2012; Sharbatkhori et al., 2015), livestock (Soba and Logar, 2008; Díaz et al., 2015; Wágnerová et al., 2016), companion animals (Sotiriadou et al., 2013; Li et al., 2015), and wild animals (García-Presedo et al., 2013). Most often, molecular diagnostics of cryptosporidiosis employs methods based on sequence analysis of the parasite genomic DNA (Iqbal et al., 2012; Díaz et al., 2015; Kaupke and Rzeżutka, 2015) including deep sequencing amplicon-based technologies (Paparini et al., 2015) or whole genome sequencing (Hadfield et al., 2015). In this article, we report an application of a next generation sequencing method (NGS) based on Illumina sequencing technology for detection and identification of *Cryptosporidium* species concurrently present in pig faecal samples.

1. Materials and methods

Two pig faecal samples (nos. 63 and 22) positive at 18 SSU rRNA (Xiao et al., 1999) and COWP-PCR (Homan et al., 1999) were analysed using the NGS method. Sample no. 63 contained a mixture of two identified *Cryptosporidium* species (*Cryptosporidium scrofarum* and *Cryptosporidium suis*), whereas sample no. 22 only revealed the presence   
of *Cryptosporidium suis*. In addition, specific amplicons at the COWP locus were obtained for both samples, and their subsequent restriction analysis using *Taq*I enzyme resulted   
in different restriction patterns than those obtained for *C. parvum*, *C. hominis,* and   
*C. meleagridis*. Subsequent sequence analysis of the COWP-PCR products showed their   
88-89% similarity to *C. meleagridis* sequences. To reveal the taxonomic properties of the putative *Cryptosporidium* species present in pig faeces, NGS was performed on the 18 SSU rRNA PCR products obtained from the tested samples (Xiao et al., 1999). The standard 18 SSU rRNA nested PCR protocol amplifies a 819-836 bp amplicon (depending on the species) suitable for restriction/Sanger sequencing analysis. Unfortunately, this amplicon size is too long for NGS approach analysis using Illumina paired end sequencing. Therefore, based on the 18S *Cryptosporidium* sequences available in GenBank,   
a new set of internal 18S primers was designed (400F and 530R) for the use with the existing F2 and R2 Xiao primers (Table 1). Two newly designed nested PCR products (I - F2/R530 and II - 400F/R2, 551bp and 443bp fragment lengths respectively) fully covered the 800 bp region of the 18 SSU rRNA gene fragment and the products obtained were suitable for paired end sequencing on a MiSeq instrument. To increase the probability of amplification of all *Cryptosporidium* species present in faeces, as well as to avoid any amplification bias (Aird et al., 2011) occurring during PCR analysis, an 18 SSU rRNA PCR was performed in triplicate followed by two independent nested reactions with the newly designed primers. Nextera XT (Illumina, USA) adaptor overhang sequences were added at the 5’end of each of the primers during oligonucleotide synthesis to allow downstream sample barcoding and sequencing on a MiSeq sequencer. The nested PCR was carried out in 20 µl volume according to a previously described protocol with the following reaction conditions: 95oC for 3min, then 30 cycles   
at 95oC for 15s, 55oC for 15s, and 72oC for 30s, and final extension at 72oC for 5min (Xiao   
et al., 1999). Amplicons were validated on 1% agarose gel, purified with Ampure XP beads (Beckman Coulter Genomics, USA) and Illumina Nextera XT barcodes were added by PCR reaction according to Illumina protocols. After barcoding, PCR amplicons were purified with Ampure XP beads.

The concentration of amplicons subjected to sequencing was estimated with a Qubit fluorimeter (Thermo Fisher, USA). Nested PCR products were pooled in equimolar ratio and sequenced in paired end mode on an Illumina MiSeq sequencer using the v3 (600 cycle) chemistry kit (Illumina, USA). The bioinformatic analysis in the CLCBio Genomic Workbench NGS pipeline **(https://www.qiagenbioinformatics.com/)** was done using default parameters. Sequencing reads were trimmed for quality (reads containing N-s and shorter than 200 nucleotides were discarded) and the remaining sequencing adaptor and PCR primer sequences were removed. Overlapping paired reads were merged into contigs and assembled *de novo* at 95% identity. . The obtained consensus contig sequences were blasted against GenBank database (NCBI).

1. Results and discussion

Illumina sequencing yielded in total 570878 reads for the analysed samples. After quality trimming of raw sequence data and their merging into pairs, 28118 sequences were obtained for amplicon 63\_I, 174240 sequences for amplicon 63\_II, 96190 sequences for amplicon 22\_I and 85274 sequences for amplicon 22\_II. *De novo* assembly of sequence reads for each (I and II amplicons) 18 SSU rRNA amplicon resulted in two (sample 63) and one (sample 22) contig sequences (Table 2). The analysis of sample 63 amplicon sequences revealed two *Cryptosporidium* species: for amplicon 63\_I *Cryptosporidium suis* 24988 reads (89.9%) and *Cryptosporidium scrofarum* 3130 reads (10.1%) and for amplicon 63\_II *Cryptosporidium suis* 157020 reads (90,1%) and *Cryptosporidium scrofarum* 17220 reads (9,9%). In the case of sample 22 both amplicons (amplicon 22\_I - 96164 reads and amplicon 22\_II – 85270 reads) showed 100% similarity to *Cryptosporidium suis*.

*Cryptosporidium* infections frequently occur in neonatal livestock. In most cases animals are infected by different host-adapted *Cryptosporidium* species. For example, in pigs, the two species *C. scrofarum* and *C. suis* are commonly detected (Suárez-Luengas et al., 2007; Kvác et al., 2009b; Lin et al., 2015; Petersen et al., 2015; Rodriguez-Rivera et al., 2016), but occasionally other species such as *C. parvum*, *C. muris*, *C. felis,* *Cryptosporidium* mouse genotype I, or rat genotype have been found (Chen and Huang, 2007; Zintl et al., 2007; Kvác et al., 2009a; Jenkins et al., 2010; Němejc et al., 2013). Cryptosporidiosis in pigs usually has a subclinical course characterised by shedding of a small amount of oocysts in faeces. In the case of asymptomatic infections, especially when they are caused by different parasite species, their identification can only be performed using molecular tools targeting the same or different gene loci (Ezzaty Mirhashemi et al., 2015). However, PCR assays targeting different regions of the *Cryptosporidium* genome have different sensitivities and specificities and are marred by preferential amplification of certain *Cryptosporidium* species (Ezzaty Mirhashemi et al., 2015). Therefore the inability to identify a parasite using one method obligates the use of another assay. This approach is frequently used for analysis of faecal samples collected from farm animals harbouring several *Cryptosporidium* species   
or genotypes, or when concurrent infections are common (Rzeżutka et al., 2014). Indeed,   
if a tested sample contains a DNA mixture originating from different *Cryptosporidium* species, then only DNA of the dominant species will be efficiently amplified and will yield   
a positive signal on the gel (Xiao, 2010). Nevertheless, the mixture of different closely related DNA sequences hinders their analysis due to mixed peaks appearing (Rieux et al., 2013).

In the tested samples, the presence of *C. suis* and *C. scrofarum* was detected at the 18 SSU RNA locus and by NGS sequencing. These samples also gave strong positive signals   
in COWP-PCR, and subsequent RFLP analysis using *Taq*I resulted in two DNA fragments   
of different size than *C. parvum*, *C. hominis*, or *C. meleagridis*. Indeed, COWP-PCR amplicons were obtained for *C. suis* DNA, but their identification was not possible as their sequences did not match any similar sequence in GenBank. To confirm this observation, other *C. suis*-positive samples were amplified using COWP-PCR, giving similar results (data not shown). Detection of *C. suis* at the COWP locus with Homan et al. primers was unexpected because this assay only permits amplification of *C. parvum*, *C. hominis*, and *C. meleagridis* DNA (Homan et al., 1999; Jiang and Xiao, 2003). Nevertheless, consistent detection   
of *C. suis* using several molecular tools (18 SSU RNA PCR and NGS sequencing) characterised by different resolution power confirms the current finding. It has previously been shown that PCR assays targeting the 18 SSU RNA gene locus provide relevant data   
on the diversity of species present in analysed samples, therefore this gene was also chosen for NGS analysis (Ezzaty Mirhashemi et al., 2015). Recently, NGS employing the 18 SSU rRNA locus has been successfully used for detection of *Cryptosporidium*-positive human and animal faecal samples (Paparini et al., 2015). In this study an alternative NGS technology based on Illumina sequencing was employed. It allowed identification of *Cryptosporidium* species in samples with low abundance of parasite DNA. Although the putative species could not be identified at the COWP locus, the amplification and sequencing of the 18 SSU rRNA gene fragment confirmed its usefulness in species characterisation. The findings of this study also imply the need for revision of the COWP-PCR primer specificity used for the identification of *Cryptosporidium* species.

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Table 1

Primer sequences used for generation of nested PCR amplicons. Illumina Nextera adaptor sequences were underlined. Newly designed primers are marked in red, whereas existing 18S SSU primers are shown in blue.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Primer | Sequence (5’- 3’) | Nucleotide position\* | Amplification stage | Product length | Reference |
| F2 | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GGAARGGTTGTATTTATTAGATAAAG | 193–218 | nested PCR  (1st reaction) | 550 bp | Xiao et al., 1999 |
| R530 | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTGCTTTAAGCACTCTAATTTTCTC | 718–743 | This study |
| 400F | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG  GTTGTTGCAGTTAAAAAGCTCGTAG | 587–611 | nested PCR  (2nd reaction) | 450 bp | This study |
| R2 | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG AAGGAGTAAGGAACAACCTCCA | 1008–1029 | Xiao et al., 1999 |

\*nucleotide position of primer binding sites was calculated based on the *Cryptosporidium parvum* 18S complete   
 reference sequence (AF093489.1)

Table 2

BLAST search results of contig sequences generated after sequencing read assembly.

|  |  |  |  |
| --- | --- | --- | --- |
| Sample  (amplicon) | *Cryptosporidium suis* | *Cryptosporidium scrofarum* | Total reads |
| 63\_I | 24988 reads (contig 1) | 3130 reads (contig 2) | 28118 |
| 63\_II | 157020 reads (contig 1) | 17220 reads (contig 2) | 174240 |
| 22\_I | 96164 reads (contig 1) | - | 96190 |
| 22\_II | 85270 reads (contig 1) | - | 85274 |