



COMPARISON OF PCR PRIMERS FOR THE IDENTIFICATION OF *CRYPTOSPORIDIUM* SPECIES AND GENOTYPES IN CALVES

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Summary

Hatalová, E., A. Valenčáková, J. Kalinová, L. Luptáková, O. Danišová & M. Húska, 2017. Comparison of PCR primers for the identification of *Cryptosporidium* species and genotypes in calves. *Bulg. J. Vet. Med.*, **20**, Suppl. 1, 154–157.

In our study we have compared three different primer sets targeting different gene regions of *Cryptosporidium parvum*. In comparison to the primers amplifying the GP60 gene, primers targeting the SSU rRNA region had a lower detection rate. Primer combination Xiao F2/R2 and VKSS F1/R2 show a detection rate of 16%, combination VKSS F1/R1 and F2/R2 a detection rate of 41.6%. The highest detection rate was achieved by primers GP60 F1/R1 and F2/R2 (58%).

Key words: calves, *Cryptosporidim*, genotypes, molecular methods, primers

INTRODUCTION

The diarrhoeal syndrome is one of the most economically significant diseases in calves under one month of age. *Cryptosporidium parvum* is detected in 85% of clinical cases (Santín *et al.*, 2004). Molecular methods for the detection of *Cryptosporidium* target various regions of the genome are used. The most common regions are, for example, the SSU rRNA region, *Cryptosporidium* oocyst wall proteins (COWP), internal transcribed spacer region (ITS), trombospondin-related adhesive protein (TRAP), beta-tubulin, the GP60 gene (Leetz *et al.*, 2007), and microsatellite markers (Feng *et al.*, 2011).

In this study, two types of reactions targeting the SSU rRNA region with the

reaction used to amplify the GP60 gene region were compared. Subsequently, percentual evaluation of the detection rate of each primer pairs was conducted.

MATERIAL AND METHODS

Faeces were collected directly from the rectum of 12 calves suffering from acute watery diarrhoea, with suspected diagnosis cryptosporidiosis. Samples were stored at –20° until DNA extraction.

One-hundred mg of faeces were homogenised and the oocysts disrupted at 6,500 rpm for 90 seconds with the addition of 0.5 mm glass beads, 1.0 mm zircon beads and 300 µL of lysis solution in Pre-

celys 24 (Bertin Technologies) homogenizer. DNA was extracted using the DNA-sorb B nucleic extraction kit (AmpliSence, Russia). Subsequently, nested PCR was used for DNA amplification.

Species determination

For the first experiment, we used the modified combination of primers according to the protocol described by Danišová *et al.* (2016): Xiao F2 (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3')/Xiao R2 (5'-CTC ATA AGG TGC TGA AGG AGT A-3') for the first reaction (819-825 bp; Fayer & Xiao, 2008) and VKSS F1 (5'-AAT TGG AGG GCA AGT CTG G-3')/VKSS R2 (5'-TAA ATA CGA AAT GCC CCC AAC-3') for the second reaction (345-355 bp; Leetz *et al.*, 2007). These primers target the SSU rRNA region.

For the second experiment, primers VKSS F1 (5'-AAT TGG AGG GCA AGT CTG G-3')/VKSS R1 (5'-CCT TCC TAT GTC TGG ACC TGG-3'; 658–662 bp) for the first reaction and primers VKSS F2 (5'-GCT CGT AGT TGG ATT TCT GTT GT-3')/VKSS R2 (5'-TAA ATA CGA AAT GCC CCC AAC-3'; 245–250 bp; Leetz *et al.*, 2007) were used, also targeting the SSU region of rRNA.

Genotype identification

For the third experiment, GP60 F1 (5'-ATG AGA TTG TCG CTC ATT ATC-3')/GP60 R1 (5'-TTA CAA CAC GAA TAA GGC TGC-3') for the first reaction (980-1000 bp) and primers GP60 F2 (5'-GCC GTT CCA CTC AGA GGA AC-3')/GP60 R2 (5'-CCA CAT TAC AAA TGA AGT GCC GC-3'; 450 bp) for the second reaction were used, targeting the GP60 gene region, according to the protocol by Iqbal *et al.* (2012).

All secondary PCR products were analysed by gel electrophoresis in 1.5% agarose gel and visualised by UV light at a wavelength of 312 nm. Subsequently, PCR products were sent for DNA sequencing. Final sequences were compared with homologous sequences from GenBank using Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Genotype determination was carried out by rules of genotyping by Xiao (2010).

RESULTS

All results are summarised in Table 1. The 12 samples from calves with suspected cryptosporidiosis, have yielded different results with each set of primers. With the primer combination Xiao F2/R2 and VKSS F1/R2, two samples out of 12 were positive (16%). With the primers VKSS F1/R1 and F2/R2, 5 samples were identified as positive (41.6%) and with primers GP60 F1/R1 and F2/R2, 7 samples out of twelve were identified as positive (58%). The samples were run for each set of primers repeatedly. The results showed that the most reliable primer for the diagnostics of *C. parvum* was GP60, with the highest percent of positive samples detected. All the positive samples from gel electrophoresis were verified repeatedly by Sanger sequencing by two independent sequencing companies.

DISCUSSION

PCR-based methods are the most sensitive techniques for detecting *Cryptosporidium* spp, although the ability to amplify the extracted DNA can be reduced by various inhibitors. Using PCR protocols for the detection of this parasite can lead to different results (Leetz *et al.*, 2007). In experiments described in this study, we have

Table 1. Detection of cryptosporidiosis with the different primers

Samples	Primers					
	XIAO+VKSS	SEQ	VKSS 1+2	SEQ	GP60	SEQ
1	+	<i>C. parvum</i>			+	IlaA17G1R1
2			+		+	
3	+	<i>C. parvum</i>	+	<i>C. parvum</i>		IlaA17G1R1
4						
5						
6			+	<i>C. parvum</i>	+	IlaA17G1R1
7					+	IlaA17G1R1
8			+	<i>C. parvum</i>	+	IlaA17G1R1
9						
10			+	<i>C. parvum</i>	+	IlaA17G1R1
11			+	<i>C. parvum</i>	+	IlaA17G1R1
12						

come to the conclusion that different primers for detection of the same species may produce different results. Primers amplifying the SSU region of rRNA show lower sensitivity and in some cases are capable of amplifying a different species, such as gregarines (unpublished results). Feng *et al.*, (2007) confirmed in their study differences in the detection with SSU rRNA primers because of the different intensity of oocyst shedding. The primers targeting the GP60 gene are used for genotyping *C. parvum*, *C. hominis* (Xiao, 2010), *C. ubiquitum* (Li *et al.*, 2014), *C. meleagridis* (Stensvold *et al.*, 2014) and *C. fayeri* (Power *et al.*, 2011), therefore, the primer targeting the GP60 region has to have a higher sensitivity and specificity than primers targeting the SSU rRNA region. Although, GP60 primer is commonly used for genotyping after primary species identification with other primers, this study shows that it has the highest rate of success in diagnostics. The findings in this study show that different PCR protocols for detecting *Cryptosporidium* are not capable to detection in all isolates, particularly the ones with a

low number of oocysts. The success of the primer combination Xiao F2/R2 and VKSS F1/R2 for the detection of *Cryptosporidium* spp. in samples has, up until now not been compared to other primer combinations. Based on these results, we have concluded, that this combination of primers is not the most optimal for the diagnostics.

CONCLUSION

In this study we have confirmed that primers targeting the GP60 gene of *C. parvum* were more specific and had a higher detection rate than primers targeting the SSU rRNA region. GP60 primers had identified 7 out of 12 samples (58% detection rate). VKSS primers detected 5 out of 12 samples (41.6%). The lowest detection rate was obtained with the primer combination Xiao F2/R2 and VKSS F1/R2 (16%). This shows that when *C. parvum* is expected in samples, primers GP60 are the most reliable, and also subtypes can be identified directly after sequencing.

ACKNOWLEDGEMENT

This work was supported by APVV No. 15/0134 and VEGA MŠ SR No. 1/0061/16.

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