Molecular and Cellular Probes xxx (2016) 1–5

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Molecular analysis of *Cryptosporidium* from cattle from five states of Peninsular Malaysia

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ARTICLE INFO

Article history:
Received 17 December 2015
Received in revised form
7 January 2016
Accepted 7 January 2016
Available online xxx

Keywords:
Cryptosporidium
Peninsular Malaysia
Cattle
Sequencing

ABSTRACT

Despite the importance of the cattle industry in Malaysia, there are very few studies of the diversity and public health significance of bovine cryptosporidiosis in this country. In the present study, we used a PCR-based approach to detect and genetically characterize *Cryptosporidium* DNA in faecal samples from a cohort of 215 asymptomatic cattle (of different ages) from six farms from five states of Peninsular Malaysia. Cattle on four of the six farms were test-positive for *Cryptosporidium*, with an overall prevalence of 3.2%. *Cryptosporidium bovis* and *Cryptosporidium ryanae* were detected in two (0.9%) and five (2.3%) samples tested; this low prevalence likely relates to the age of the cattle tested, as most (73%) of the samples tested originated from cattle that were ≥2 years of age. Future studies should investigate the zoonotic potential of *Cryptosporidium* in pre-weaned and weaned calves in rural communities of Malaysia.

1. Introduction

Cryptosporidium species are obligate intracellular protistan parasites that can infect a wide range of animals and human hosts worldwide [1]. Cattle are considered to represent key animal reservoir hosts of *Cryptosporidium*. Importantly, cryptosporidiosis is recognized as one of the major causes of neonatal calf diarrhoea, resulting in weight loss and growth retardation, morbidity and, in severe cases, death, leading to considerable economic losses [2,3]. Furthermore, several foodborne and waterborne outbreaks of human *Cryptosporidium* infections have also been attributed to contamination of food produce and drinking water by cattle manure [2,4].

The traditional approach for the diagnosis of infection relies on the microscopic detection of cysts or oocysts in stool samples, but this approach is unable to distinguish the different *Cryptosporidium* taxa based on morphometric or other phenotypic characteristics, due to lack of differentiating morphological features [5,6]. Therefore, molecular tools, such as polymerase chain reaction (PCR)-based methods have been employed, targeting taxonomically informative loci to circumvent this limitation [2,7]. Currently, at least 23 different species of *Cryptosporidium* and more than 70 genotypes have been recognized, with new genotypes continually being identified by molecular means [2]. To date, seven species have been recorded in cattle, which include *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae*, *Cryptosporidium andersoni* and *Cryptosporidium suis*, as well as two genotypes of *Cryptosporidium* (i.e. pig genotype II and a new *C. suis*-like genotype) [8,9]. Given this diversity of *Cryptosporidium* species in cattle and differing zoonotic potential [1,2], it is important to be able to estimate their prevalence and distribution (temporally and spatially) in particular geographic regions using molecular tools.

In Malaysia, the cattle industry is one of the key components of the agricultural sector, providing gainful employment and producing high quality protein (red meat) and milk for human population. In 2014, Malaysia's ex-farm value of beef was RM 1.25 billion and additional RM 150.54 million for milk, contributing to 9.1% of the total national output of livestock products [10]. Despite the importance of the cattle industry, there are only two published studies of *Cryptosporidium*/cryptosporidiosis of cattle in Malaysia

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http://dx.doi.org/10.1016/j.mcp.2016.01.002
0890-8508/

using microscopic methods [11,12], and two others using molecular techniques [13,14]. Given this lack of information, further studies of different age groups of cattle from different geographical areas are needed to obtain more information on prevalence, distribution and health and economic impact of bovine cryptosporidiosis in Malaysia. Therefore, in the present study, we employed a PCR-based approach targeting genetic markers in the small subunit of ribosomal RNA (*SSU*) and 60 kDa glycoprotein (*gp60*) [15] to genetically characterize *Cryptosporidium* in faecal samples from a cohort of 215 asymptomatic cattle from six farms from five states of Peninsular Malaysia. The aim was to evaluate the species and/or genotypes that they harbour and whether the infections might have zoonotic potential.

2. Materials and methods

2.1. Ethical consideration

The study protocol was approved by the Ethics Committee of the University Malaya Medical Center, Malaysia (MEC Ref. No. 896.36). Permission for the study to be conducted on animal farms was obtained from owners prior to sample collection.

2.2. Faecal sample collection

A total of 215 faecal samples were collected from cattle from six different farms located in east coast (Farm A, Kuantan, Pahang state), northern (farm B, Sungai Siput, Perak state), Central (farm C, Serdang, Selangor state; farm D, Jerantut, Pahang state) and southern (farm E, Jelai Gemas, Negeri Sembilan state; farm F, Ayer Hitam, Johor state) parts of Peninsular Malaysia (Fig. 1). The six farms belong to the Department of Veterinary Services, Ministry of Agriculture and Agro-Based Industry, Malaysia. Faecal samples were collected rectally from individual animals and kept at 2 to 8 °C immediately after sampling, and frozen at −20 °C for subsequent DNA isolation and molecular testing.

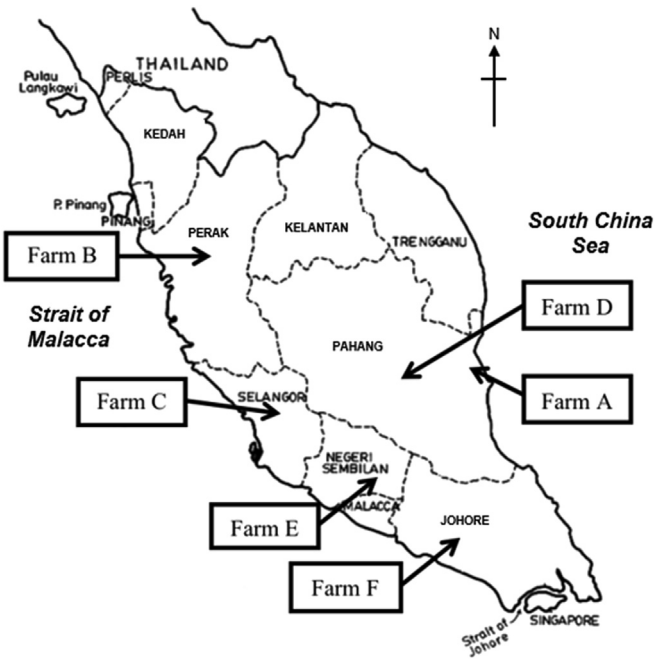

Fig. 1. Locations of farms A-F in Peninsular Malaysia from which bovine faecal samples were collected for PCR-based testing for *Cryptosporidium*.

2.3. Isolation of genomic DNA from faecal samples and PCR amplification

Genomic DNA was isolated from each faecal sample using the PowerSoil DNA Isolation Kit (MoBIO, USA), according to the manufacturer's protocol, and then frozen at −20 °C until use. Each genomic DNA sample was subjected to nested PCR, for *Cryptosporidium* employing regions (designated p*SSU* and p*gp60*) within the small subunit nuclear ribosomal RNA and 60 kDa glycoprotein genes.

For human-infective *Cryptosporidium*, primary PCR was carried out using primers gp15-ATG (forward: 5′-ATGA-GATTGTCGCCTCATTATC-3′) and gp15-STOP (reverse: 5′-TTACAA-CACGAATAAGGCTGC-3′) [16], followed by a secondary reaction to amplify a portion of the *gp60* gene (called p*gp60*; 250–380 bp) using primers gp15-15A (forward: 5′-GCCGTTCCACTCAGAGGAAC-3′) and gp15-15E (reverse: 5′-CCACATTACAAATGAAGTGCCGC-3′) [17]. Both primary and secondary PCRs were performed in a volume of 50 μL containing 3.0 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of Go*Taq* (Promega) DNA polymerase in standard PCR buffer (Promega, USA). Primary amplification of p*gp60* utilized the cycling protocol which included an initial cycle of 94 °C for 5 min (initial denaturation), followed by 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 1 min (extension), with a final extension of 72 °C for 10 min. From 1 μL of primary amplicon, p*gp60* was amplified using a cycling protocol of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

For the amplification of p*SSU* for *Cryptosporidium*, nested PCR was performed in a 50 μL volume containing 2.0 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 25 pmol of each oligonucleotide primer and 1.25 U of Mango*Taq* polymerase in a standard buffer (Bioline, USA). Primary reaction was performed using primers XF2 (forward: 5′-GGAAGGGTTGTATTTATTAGA-TAAAG-3′) and XR2 (reverse: 5′-AAGGAGTAAGGAACAACCTCCA-3′) [18], followed by a nested amplification of a portion of SSU gene (p*SSU*) using primer set p*SSU*f (forward: 5′-AAAGCTCGTAGTTG-GATTTCTGTT-3′) and p*SSU*r (reverse: 5′- ACCTCTGACTGTTAAATA-CRAATGC-3′) [19]. Primary amplification was carried out at 94 °C for 5 min (initial denaturation), followed by 30 cycles of 94 °C for 45 s (denaturation), 45 °C for 2 min (annealing) and 72 °C for 1.5 min (extension), with a final extension of 72 °C for 10 min. From 1 μL of primary amplicon, a secondary amplification was performed using a cycling protocol of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 10 min.

2.4. DNA sequence analysis

Secondary PCR products were purified using ExoSAP-IT® (Fermentas, USA), according to the manufacturer's instructions, and then subjected to direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA) using the forward and reverse primers employed in secondary PCR. The quality of each sequence was assessed based on the corresponding chromatogram, and sequences were compared with reference sequences using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

3. Results and discussion

All 215 genomic DNA samples derived from cattle faecal samples from six farms in five different states of Malaysia were subjected to the genetic analysis of *Cryptosporidium*. Although p*gp60* was not

Please cite this article in press as: N.J. Yap, et al., Molecular analysis of *Cryptosporidium* from cattle from five states of Peninsular Malaysia, Molecular and Cellular Probes (2016), http://dx.doi.org/10.1016/j.mcp.2016.01.002