Genetic Characterization of *Cryptosporidium parvum* Isolates from Cattle in Portugal: Animal and Human Implications

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Cryptosporidium parvum (APICOMPLEXA: COCCIDIA) is an opportunistic protozoa with a worldwide distribution. The parasite causes diarrhea in humans and in livestock - mainly neonatal diarrhea with high morbidity, variable mortality and economic losses. In calves, diagnosis is impaired by the presence of other agents such as *Rotavirus*, *Coronavirus*, *E. coli* K99, and *Salmonella* spp. Several waterborne cryptosporidiosis outbreaks have been reported [5]. *C. parvum* is associated with either innate or acquired immunodeficiency. Zoonotic transmission does occur and several genotypes of *C. parvum* are well known. PCR-RFLP is one of the techniques applied to study genetic polymorphisms. In Portugal, the prevalence of bovine cryptosporidiosis ranges between 12% in the North and 44% in the South [Pereira da Fonseca, I.M. & Cannas da Silva, J. 2000. Bovine cryptosporidiosis: epidemiological study in Portugal. Proceedings XXI Congresso Mundial de Buiatria, Punta del Este, Uruguai, 4-8 Dez., 2000]. The objectives of this work were: (i) to study the prevalence of cryptosporidiosis in cattle from several age groups at "Alentejo" and "Ribatejo e Oeste" regions of Portugal by EIA-ELISA; (ii) to detect oocysts in animal drinking water, in order to evaluate its importance as a risk factor for cryptosporidiosis; (iii) to determine the genotype of *C. parvum* isolates and (iv) to evaluate the importance of the obtained data in Public Health.

MATERIALS AND METHODS

Epidemiological study - A total of 553 faecal samples from cattle were collected between January 1996 and February 1998. At "Alentejo", 456 samples were obtained and at "Ribatejo e Oeste" the number of samples was 97. Twelve age groups were defined: Group 1 (1-7 days old), group 2 (8-15), group 3 (16-24), group 4 (25-32), group 5 (33-66), group 6 (67-100), group 7 (101-134), group 8 (135-180), group 9 (181-270), group 10 (271-360), group 11 (361-720) and group 12 (721-1440). The EIA-ELISA was performed using the kit "Melotest *Cryptosporidium* Ag" from Redifarma, Spain. Positive results for *C. parvum* were confirmed by faecal smears stained with modified Zielh-Neelsen.

Genotyping of *C. parvum* isolates - Genomic DNA was extracted from 186 samples (128 positives and 58 negatives) and purified by two methods: guanidinium thiocyanate/silica and proteinase K/heat followed by ethanol precipitation. Two pairs of primers were used in the PCR [3, 4]. One of the primers was directed to a 550 bp region of the *Cryptosporidium* oocyst wall protein (COWP) gene [*Cry-15*: 5'-GTA GAT AAT GGA AGA GAT TGT G-3' and *Cry-9*: 5'-GGA CTG AAA TAC AGG CAT TAT CTT G-3'] (94°C/50", 55°C/30", 72°C/45"x45 and 72°C/10") and other to a 506 bp region of the thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1) gene [*El-E*: 5'-GGA TGG GTA TCA GGT AAT AAG AA-3' and *El-W*: 5'-CAA TTC TCT CCC TTT ACT TC -3'] (94°C/50", 50°C/30", 72°C/45" x40 and 72°C/10"). The PCR-RFLP was performed with the restriction enzyme *RsaI*. The PCR amplified DNA and the restriction fragments were analysed on a 2% agarose electrophoresis gel stained with ethidium bromide and visualized by UV transillumination.

Oocysts detection in animal drinking water - Between May 1998 and January 2000, 11 samples of 10 litres each were collected at three dairy farms both from the tap and the calf bucket. Method 1622 of the United States Environmental Protection Agency (US EPA) was used to detect *Cryptosporidium* oocysts [6]. Filtration was performed with Envirocheck capsules (Gelman); for the immunomagnetic separation of the oocysts, the kit "Dynabeads anti-*Cryptosporidium* (Dynal) was used. The pellet obtained was stained with monoclonal antibodies (kit Monofluo *Cryptosporidium* - Sanofi) and 4',6-diamidino-2-phenylindole (DAPI) was used to evaluate oocysts viability.

RESULTS AND DISCUSSION

A global prevalence of 23.3% (129/553) was found being 21.1% (96/456) in "Alentejo" and 34.0% (33/97) in "Ribatejo e Oeste" regions using the EIA-ELISA technique. Group 3 with 47.2% (17/36) and group 2 with 41.7% (53/127) were the age groups which showed higher prevalences of cryptosporidiosis. Infections of 9.5% (2/21) and 10.8% (4/37) were detected in calves (1-7-days old) and adults, respectively. These animals were asymptomatic carriers.

The amplification results of COWP and TRAP-C1 genes by PCR in the two studied regions showed that 58.6% (14/24) of the isolates were positive for TRAP-C1 from which 71.4% were from "Ribatejo e Oeste" and 40.0% from "Alentejo". For COWP, the genomic DNA was amplified in 25.0% (6/24) of the isolates; 14.3% were from "Ribatejo e Oeste" and 40.0% from "Alentejo". TRAP-C1+ COWP were detected, in 16.7% (4/24) of the samples; 14.3% were from "Ribatejo e Oeste" and 20.0% were from "Alentejo". The sample sizes were too small to allow meaningful statistical analysis on geographical differences.

After PCR-RFLP of the 24 isolates, only the cattle genotype was found (Fig. 1).

Fig 1. Amplification and *RsaI* restriction length polymorphisms of the TRAP-C1 and COWP genes. Lanes 1, 9, 16 - 100 bp ladder; lanes 2 and 3 - PCR products of COWP gene: 550 bp; lanes 4 to 8 - hydrolysis fragments of COWP gene with *RsaI* (410, 106 and 34 bp); lanes 10 and 11 - PCR products of TRAP-C1 gene (506 bp); lanes 12 to 15 - hydrolysis fragments of TRAP-C1 gene with *RsaI* (455 and 51 bp).

The human genotype has different polymorphism patterns - TRAP-C1: 314, 114 and 51 bp and COWP: 285, 125, 106 and 34 bp [2].

Analysis of animal drinking water showed that 6 out of 11 (55.0%) samples were positive. At farms A and B, 50.0% (2/4) of

the samples had *Cryptosporidium* oocysts. All the samples were collected from the tap. At farm C, 66.7% (2/3) of the samples were positive; one from the calf bucket and the other from the tap. The number of *Cryptosporidium* oocysts detected in the water samples ranged between one to nine. Oocyst recovery rates for positive controls were 22.5% and 13.0%. The results of the viability study with DAPI on the six positive samples were negative. *Cryptosporidium* oocysts were detected in the animal drinking water of the three farms in every season evaluated [1].

In conclusion, the prevalence of bovine cryptosporidiosis was high at the two studied regions. The only genotype found was the bovine type, which is common to animals and man, suggesting that *C. parvum* transmission is not necessarily a zoonosis, although zoonotic transmission can also occurs. Bovine play an important role as reservoirs for human cryptosporidiosis and also as a source of environmental contamination. Although viability studies with DAPI were not conclusive, oocysts were observed in water used for calves. This fact can contribute for the spread of animal cryptosporidiosis, environmental contamination and public health risks. Due to the small number of oocysts found in the water samples, determination of the isolates' genotype was not possible. Cryptosporidiosis study at Portugal needs to be pursuit by genotyping more isolates from faecal, animal drinking water and farm effluent samples using other molecular markers.

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