Genotyping of *Cryptosporidium* spp. isolated from human stool samples in Switzerland

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SUMMARY

In a study to estimate the frequency of *Cryptosporidium* infections in Switzerland, stool samples from patients found to be positive for *Cryptosporidium* spp. by modified Ziehl–Neelson staining and fluorescence microscopy were used for genotyping experiments. With 9 of 12 samples, DNA extraction and subsequent genotyping was successful. All *Cryptosporidium*-isolates belonged to the bovine genotype. In one stool sample, two strains of *Cryptosporidium* were demonstrated, suggesting a mixed infection. In comparison with reference strains from calves, one of the isolates showed a full sequence identity and the other a similarity of 97·5%. The fact that only bovine genotypes were detected suggests, that cryptosporidiosis must primarily be considered as a zoonotic disease in Switzerland. This is in contrast to other countries, where the human genotype of *C. parvum* was shown to dominate the epidemiological situation. The results of our study are supported by the previous finding, that two of the analysed strains originated from patients who used to consume raw milk or raw cream, a known risk factor for cryptosporidiosis.

INTRODUCTION

Cryptosporidium is a protozoan parasite which infects mainly the apical region of epithelial cells lining the gastrointestinal tract or associated organs such as the biliary tract of vertebrates and sometimes the respiratory tract [1]. Prior to the 1980's, infections with species of the genus *Cryptosporidium* were considered to be rare in animals. In humans, *Cryptosporidium* was thought to be an opportunistic pathogen in immunocompromised individuals. Nowadays however, it is apparent that the species *Cryptosporidium parvum*

is responsible for zoonotic and anthroponotic infections in both urban and rural settings worldwide. The parasite causes self-limiting diarrhoea in immunocompetent humans, and severe, persistent or chronic life-threatening diarrhoea in immunocompromised patients, particularly in those suffering from the acquired immunodeficiency syndrome, AIDS [1–3]. Cryptosporidial infections are now considered as one of the most common non-viral and non-bacterial causes of diarrhoea in humans and livestock [4]. Symptoms are relatively non-specific, namely nausea, acute onset of profuse and watery diarrhoea, lack of appetite, vomiting, weight loss, abdominal pain and flu-like symptoms [1, 5]. Infections are transmitted faecal-orally (ingestion or inhalation), either directly

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from person-to-person, from animal-to-person, or indirectly through contaminated vectors like water or food [5, 6].

Various *Cryptosporidium* species have now been recorded in over 170 different host animals originating from over 50 countries [5]. Taxonomically, the 11 following species are known: *C. parvum*, *C. wrairi*, *C. meleagridis*, *C. saurophilum*, *C. felis*, *C. canis*, *C. baileyi*, *C. muris*, *C. andersoni*, *C. serpentis*, *C. nasorum* [3, 7, 8]. *Cryptosporidium parvum* has been considered to be the only species that infects immunocompetent humans [5, 9]. However, this classification, based mainly on morphology, biology and genetic information, is still changing and more data are needed before the taxonomic status of the current species within the genus *Cryptosporidium* can be clearly defined. Genetic data has shown that *C. parvum* is not a uniform species. Eight different genotypes within *C. parvum* have been described: human, cattle, pig, mouse, kangaroo, dog, ferret and monkey genotype [10]. To date, with the exception of the cattle genotype, each of these types occur only in their respective hosts, suggesting host specificity [10]. There are two different genotypes of *C. parvum* infecting immunocompetent humans and cattle; a human and a bovine genotype respectively, with the later genotype being zoonotic [3, 11–14]. As the human genotype appears to be host specific, this could result in a higher infectivity of the human genotype compared to the cattle genotype in humans [3, 15–17].

In Switzerland, detection of *Cryptosporidium parvum* is not routinely done in laboratories of clinical microbiology and there is no obligation to communicate isolations of this pathogen to health authorities. For these reasons, frequency of cryptosporidiosis and sources of infection were only poorly known. In the past 15 years, only three epidemiological studies in the context of cryptosporidiosis were performed. Initial research work estimated the frequency of *Cryptosporidium* spp. in faecal samples of immunocompetent children with gastroenteritis [18]. In the case of toddlers, other authors have shown person-to-person contacts as the most important way of transmission [19, 20]. Finally, a most recent study revealed data about the frequency of *Cryptosporidium* spp. in Switzerland. Faeces from 6435 ambulatory and hospitalized diarrhoeal patients were screened for *Cryptosporidium* spp. over the period of one year in two laboratories. In total, 13 patients with cryptosporidiosis were detected which results a frequency of 0·2%. A crude estimate of the total number of cryptosporidiosis cases which would have to be expected yearly in Switzerland if routine diagnostics for this organism were done in the same frequency as for salmonella and campylobacter and if laboratory isolates were reported to the Federal Health Authorities revealed a total value of 340 yearly cases in Switzerland, which results a morbidity of 4·85 per 100 000 persons of the Swiss population [21]. Twelve isolates positive for *Cryptosporidium* spp. from this survey were genotyped in the present study which allowed a more precise insight into the epidemiological characteristics of cryptosporidiosis in Switzerland.

MATERIALS AND METHODS

Stool samples positive for *Cryptosporidium* spp.

Genotyping included *Cryptosporidium* spp. isolates from 12 stool samples collected in a former study [21]. Additionally, three faecal samples positive for *Cryptosporidium* spp. from Swiss calves were kindly supplied by the Cantonal Laboratory Basel-Landschaft and included into the typing experiments.

Genotyping

Human and calf stool samples containing oocysts of *Cryptosporidium* spp. were stored at 4 °C in 2·5% potassium dichromate solution until they were used. The oocysts were purified by a sucrose and Percoll gradient method [22]. DNA was isolated from the purified oocysts as previously described [23]. A modified two-step nested-PCR protocol, previously described [24], was used to amplify the HSP70 gene [25]. PCR products were purified using Qiagen spin columns (QIAquick Gel Extraction Kit, Germany), and sequenced by a Swiss company (Microsynth GmbH).

Analysis of sequence data

The sequences were aligned with the ClustalW sequence alignment program [26] and phylogenetic analysis of HSP70 sequence data was conducted using the Phylogeny Inference Package PHYLIP 3.5c [27]. Distance-based analyses were conducted using the formula for Kimura's Two-Parameter Model (for estimating the number of nucleotide substitutions per site between sequences). Phylogenetic trees were constructed using the Unweighted Pair-Group Method (UPGMA) algorithms available in PHYLIP 3.5c. Phylograms were drawn using the TreeView 1.6.1 program [28]. Supplemental HSP70 sequences were
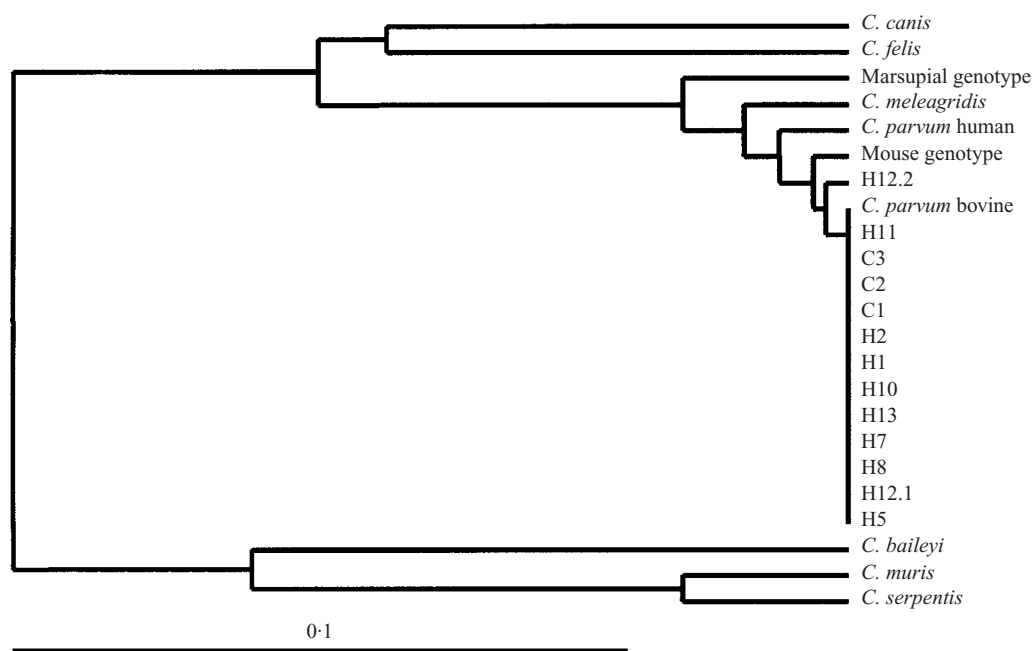
Fig. 1. Phylogram of Kimura's distance generated from HSP70 sequence information (using UPGMA) with the *Cryptosporidium* spp. isolates from human ('H') and from calf stool samples ('C') and with different references of *Cryptosporidium* spp.

obtained from GenBank: a bovine *C. parvum* isolate (AF221528), *C. canis* (AF221529), a mouse genotype (AF221530), a marsupial genotype (AF221531), a human genotype (AF221535), *C. meleagridis* (AF221537), *C. felis* (AF221538), *C. baileyi* (AF221539), *C. serpentis* (AF221541) and *C. muris* (AF221542).

RESULTS AND DISCUSSION

Genotyping experiments were successful with 9 of total 12 (75%) *Cryptosporidium* spp. isolates from human stool samples. All HSP70 sequences belonged to the bovine genotype of *C. parvum*, as shown in Figure 1. Three human isolates could not be amplified although several attempts to genotype have been undertaken. Surprisingly, in one sample (H12), repeated PCR-reactions revealed two different genotypes: the bovine *C. parvum* genotype (H12.1) and another (H12.2). Sequence identities of sample H12.2 with the reference genotypes were as follows: with the bovine genotype of *C. parvum*: 97.5%; with the human genotype of *C. parvum*: 95.3% and with the mouse genotype: 96.4%. All three isolates from calves demonstrated clearly the bovine genotype of *C. parvum*. The inability to genotype the remaining three *Cryptosporidium* spp. isolates from human stool samples was probably due to the fact that only very small amounts of faecal materials was available. Furthermore, the extraction technique used resulted in low DNA yields. All sequences were submitted to NCBI GenBank (accession numbers AY151404–AY151416).

Humans are susceptible both to the human and to the bovine genotype of *C. parvum*, and mixed infections have been shown to occur [29–31]. In Australia, the majority of sporadic cases (85%) were caused by the human genotype, suggesting that infection with anthroponotic parasites played a more important role than infection with zoonotic parasites in these individuals [3, 32, 33]. In contrast, the *C. parvum* bovine genotype was responsible for more sporadic human cases in the UK (57%) than the *C. parvum* human genotype [3, 17]. Interestingly, 14 investigated outbreaks in North America were dominated by the human genotype of *C. parvum* (human genotype: 71%, cattle genotype: 29%) [3].

Genotyping in the present study exclusively revealed the bovine genotype of *C. parvum*. Three of the nine persons with genotyped *Cryptosporidium* spp. isolates had travelled abroad prior to onset of symptoms and the remaining six persons were infected in their home country. Out of those, two persons had consumed raw milk and raw cream, foods known to be risk factors for cryptosporidiosis [21].

As the prevalence of *C. parvum* in Swiss calves within the first 3 months of life is very high (16·8%) [34], we came to the conclusion that the bovine genotype of *C. parvum* must be the most important cause of sporadic cryptosporidiosis in Switzerland with cattle as the probable primary reservoir. This conclusion is also supported by the above mentioned consumption of raw milk and raw cream by two patients.

The reported predominance of the bovine genotype is interesting in relation to the previous findings that showed person-to-person transmission as a major risk factor in the same area of Switzerland [19, 20]. The findings can be reconciled as firstly, that both human and bovine genotypes may be transmitted from person-to-person [35]. Secondly, one third of the patients where the genotyped strains originated from had contact with symptomatic persons prior to onset of symptoms [21].

For the prevention of cryptosporidiosis, personal hygiene is of central importance. However, the high prevalence of *Cryptosporidium* spp. in cattle and the predominance of the bovine genotype in cases of human cryptosporidiosis also requires particular measures of food hygiene which are the appropriate heating of raw milk and products thereof prior to consumption. This measure, preventing infections with various pathogens transmittable by raw milk, is officially recommended by the Swiss Federal Health Authorities [36].

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