CRYPTOSPORIDIUM: FROM LABORATORY DIAGNOSIS TO SURVEILLANCE AND OUTBREAKS

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Summary:

The burden of disease caused by the protozoan parasite *Cryptosporidium* is unknown. However, routine laboratory diagnosis and surveillance enables the basic epidemiology to be described, changes to be monitored and under-ascertainment to be measured. Although the two main species involved in human disease in developed countries, *Cryptosporidium parvum* and *Cryptosporidium hominis*, have differing epidemiologies and risk factors, national surveillance is generally from isolates identified to the genus level only. Enhancing the data by typing, at least to identify the isolates to the species level, removes some of the noise generated and better identifies the risks than when reports are not species-specific. This level of identification is also valuable for outbreak investigations, but further investigation of the population genetics of *C. parvum* and *C. hominis* is required for the development of more readily applied subtyping tools.

KEY WORDS : *Cryptosporidium*, diagnosis, surveillance, outbreaks, genotyping.

Although the Apicomplexan *Cryptosporidium* was first described at the beginning of the twentieth century (Tyzzer, 1907), human disease (cryptosporidiosis) was first identified almost three quarters of a century later. Two reports were published in 1976: one based on the findings of histological examination of jejunal biopsy from a severely dehydrated immunosuppressed adult male patient with chronic watery diarrhoea (Meisel *et al.*, 1976) and the other on examination of rectal biopsy from an otherwise healthy female child with watery diarrhoea, abdominal pain and vomiting (Nime *et al.*, 1976). The first patient recovered after withdrawal of immunosuppressive treatment and subsequent restoration of T-cell count and function and the child recovered spontaneously after two weeks. In the 1970s, *Cryptosporidium* was beginning to be recognised as a cause of gastro-intestinal disease ("scours") in neonatal calves (Panciera *et al.*, 1971; Pohlenz *et al.*, 1978) and during the 1980s, awareness of the potential of this parasite to cause disease in humans increased largely as a result of the AIDS epidemic. *Cryptosporidium* was widely regarded as an opportunist zoonotic infection causing severe and often fatal disease in AIDS patients (Ma & Soave, 1983; Current *et al.*, 1983), who were then mainly urban dwelling, adult male homosexuals. However, other immunocompromised patients and, conversely, otherwise healthy people, including veterinary workers and particularly veterinary students, were also diagnosed with cryptosporidiosis (Jokipii *et al.*, 1983; Current *et al.*, 1983). This presented an epidemiological conundrum: was the disease an opportunist; was it a zoonosis; what were the transmission routes; which other groups might be at risk? Similarly, there were diagnostic questions: had the difficulties in laboratory diagnosis led to gross under-ascertainment of this pathogen?

Early epidemiological studies demonstrated an urban cycle of infection in otherwise healthy people, and confirmed the importance of day care centres and person-to-person spread (Casemore & Jackson, 1983). The validity of the description of cryptosporidiosis solely as an opportunistic zoonosis was doubted (Casemore & Jackson, 1984), while several independent studies during the 1980s worldwide showed cryptosporidiosis as a common cause of self-limiting gastroenteritis in otherwise healthy people, particularly children (Casemore, 1990; Palmer & Biffin, 1990). The first outbreak which led to the recognition of *Cryptosporidium* as a waterborne pathogen occurred in Texas, USA in 1984, involving 79 cases with diarrhoea of which 47 were laboratory confirmed as *Cryptosporidium*. The public water supply, which came from a well contaminated with sewage, was the implicated vehicle (D'Antonio *et al.*, 1985). It thus became evident that the epidemiology of cryptosporidiosis is complex, involving both direct and indirect transmission from both animals and humans, enabled by the robust nature of the transmissive stage of the parasite, the oocyst. However, microbiological evidence for the sources of infection was not possible until suitable typing methods were established.

Laboratory diagnosis of cryptosporidiosis is traditionally by tinctorial or fluorescent staining of faecal smears followed by microscopical examination, or more recently by the use of copro-antigen detection kits, such as immunofluorescent antibody stains for microscopy, enzyme linked immunosorbent assays (ELISAs) or immunochromatographic tests. The analytical sensiti-

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vity of these methods is generally in excess of 10^4 oocysts per gram faeces (opg), depending on faecal consistency (Anusz *et al.*, 1990; Weber *et al.*, 1991), although immunofluorescence microscopy offers improved sensitivity (Arrowood, 1997). The oocyst is the diagnostic target, and none of these methods is capable of differentiating *Cryptosporidium* species. A variety of detection methods are employed and, in addition, laboratory selection criteria for testing vary: in the UK it is recommended that all first time acute faecal samples from community cases of diarrhoea are tested for *Cryptosporidium* (Health Protection Agency, 2007), and there is generally good, but not total, compliance with this (Chalmers *et al.*, 2002a). Elsewhere, laboratory testing is often restricted to AIDS patients. Surveillance strategies for *Cryptosporidium* also vary: in some countries the laboratory diagnosis is statutorily notifiable at a national level, for example in Ireland, Finland, Germany and Sweden, while in others data are collected on a voluntary reporting basis, for example in the UK and the Netherlands. In France there is a reporting network of sentinel laboratories. Differences also occur locally (Chalmers *et al.*, 2002a), but voluntary reporting in the UK is widespread and goes back to 1983. Between 1983 and 1989 reporting increased as laboratory testing became more common, and since 1990 reports to the Health Protection Agency's Centre for Infection (CfI) show a mean annual incidence of 8.9 cases per 100000 population, with all ages affected but most reported in children under five years (Nichols *et al.*, 2006a). There is an annual bimodal distribution with peaks in the spring and autumn, but since 2001 there has been an overall reduction in the number of reports in the first part of the year, linked to the beneficial impact of The Water Supply (Water Quality) (Amendment) Regulations 1999 (Sopwith *et al.*, 2005; Lake *et al.*, 2007). The risk factors for cases in the second half of the year are unclear but may be linked to travel and recreational water use. The total disease burden from *Cryptosporidium* is unknown: a study of infectious intestinal disease in England in 1995 suggested that, when traditional diagnostic methods are used, one in every 7.4 cases are diagnosed and reported (Adak *et al.*, 2002). Further infections would be identified by the use of more sensitive diagnostic tests, such as PCR-based methods (Amar *et al.*, 2007).

These national variations in laboratory testing and surveillance strategies will have an impact on the number of cases and indeed outbreaks identified as those recognized in one country might be missed elsewhere. This is illustrated well in a review of cryptosporidiosis surveillance and waterborne outbreaks in Europe (Semenza & Nichols, 2007) where cryptosporidiosis is notifiable at European Union level. Over 70 % of reports were from the UK, reflecting the high degree of laboratory testing and reporting, and the highest incidence was in Ireland where cryptosporidium was made a reportable diagnosis in 2004. Reports may also be collected as part of surveillance for acute gastroenteritis linked to food poisoning or for outbreaks. For example, in the USA a voluntary national outbreak surveillance system began in 1971, and initially included drinking waterborne outbreaks, expanded in 1978 to include recreational water outbreaks and in 1999 those related to water not intended for drinking, exposures in occupational settings and commercially bottled water. The system is continually updated and the most recent summary, covering the period 2002 to 2003, also includes outbreaks linked to contaminated ice and beverages, drinks machines and water at the point of use (Anon, 2006; Yoder *et al.*, 2007).

Structured surveillance for outbreaks of gastro-intestinal disease has been undertaken in England and Wales since 1992 (Wall *et al.*, 1996), and indicates that about 10 % of reported cases of cryptosporidiosis are part of identified outbreaks (Nichols *et al.,* 2006a). Between 1983 (when surveillance for *Cryptosporidium* began) and 2005, a total of 151 *Cryptosporidium* outbreaks involving 9893 cases illness were reported to CfI, involving many vehicles for infection, including public water supplies (56 outbreaks), private water supplies (6), swimming pools (44), other recreational waters (7), animal contact and farms (19), food (4), person-to-person spread (10) and unknown (5). *Cryptosporidium* clearly plays a particular role in waterborne disease, facilitated by many inherent biological features, reported elsewhere (Karanis *et al.*, 2007). In a review of 89 outbreaks of waterborne disease involving 4,321 cases in England and Wales (Smith *et al.*, 2006), *Cryptosporidium* was the causative agent in 69 %. The strength of association with water is measured by the robustness of epidemiological and environmental evidence (Tillet *et al.*, 1998), although the contamination event may be missed in terms of sampling by the time an outbreak is recognised. This is particularly important for the investigation of *Cryptosporidium* where the incubation period between exposure and the development of symptoms is long (up to two weeks) and the interpretation of indicator data (microbial and water treatment process data, including turbidity) requires careful interpretation. Additionally, sampling for and laboratory identification of waterborne pathogens is difficult, particularly for *Cryptosporidium*. In an analysis of public supply outbreaks in the USA, an aetiological agent was not determined in 41 % (Craun *et al.*, 2002). Even where continual monitoring for *Cryptosporidium* is in place, as it has been at some water treatment plants in the UK for nearly a decade, interpretation of the results is complex: outbreaks have occurred where low numbers of oocysts have been detected (Anon, 2006), and high numbers of oocysts do not necessarily lead to increased disease (Hunter *et al.*, 2000). This is because the dynamics of an outbreak are multi-factorial, and the

behaviour and herd-immunity of the exposed population and the infectivity of the oocysts for man are both important.

Human disease has been traditionally attributed to *Cryptosporidium parvum* but it was apparent from both the early epidemiological questions raised regarding the zoonotic status and transmission of the organism that variants occurred. Animal infectivity studies in the 1980s demonstrated that infective dose sizes and clinical responses varied between isolates (Fayer & Ungar, 1986; Pozio *et al*., 1992) and human infectivity studies have demonstrated differences in $ID_{50,}$ attack rate, pre-patent period, duration of disease, and frequency of stools between three different *C. parvum* isolates (IOWA, TAMU and UCP) (Okhuysen *et al*., 1999). Phenotypic analysis focussing on protein, antigenic and isoenzyme diversity showed that differences were consistent with other biological and/or epidemiological data and "animal types" and "human types" were suggested by McDonald and Awad-el-Kariem in 1995. The limitation of these phenotypic methods was the huge numbers of oocysts required, which hampered their widespread use. Analysis of the *C. parvum* genome at a repetitive DNA sequence showed genetic heterogeneity within the species (Ortega *et al*., 1991) but also required enormous numbers of oocysts. It was the amplification provided by PCR that facilitated further exploration of differences at the genomic level (Laxer *et al*., 1991). Throughout the 1990s genetic analysis of human isolates consistently identified two distinct *C. parvum* types, one variously referred to as the "cattle" genotype, genotype 2 or C and the other as the "human" genotype, genotype 1 or H. In 2002, Morgan-Ryan *et al.* proposed that the "human" genotype was a separate species, *Cryptosporidium hominis* n. sp., on the basis of evidence conforming to criteria shown below. At the time of writing, 16 *Cryptosporidium* species are currently recognised, defined according to the criteria agreed at the 6th Meeting on Molecular Epidemiology and Evolutionary Genetics in Infectious Disease in Paris, France (Xiao *et al*., 2004): *C. parvum, C. hominis, C. felis, C. canis, C. wrairi, C. varani, C. suis, C. bovis, C. andersoni, C. muris, C. serpentis, C. galli, C. meleagridis, C. fayeri, C. macropodum* and *C. baileyi*. The four basic requirements for the naming of *Cryptosporidium* species are:

1. Morphometric study of oocysts and, if possible, sporozoites.
2. Multi-locus genetic characterisation by nucleotide sequence analysis of well studied genes or non-coding regions. The ssu rRNA gene is usually included.
3. Demonstration of natural and, if possible, experimental, host specificity.
4. Compliance with the International Code of Zoonotic Nomenclature.

Analysis of the ssu rRNA gene by DNA sequence analysis is regarded as the benchmark against which identifications made at alternative loci can be compared. The advantages of the ssu rRNA gene are two-fold. First, it is sufficiently conserved so that primers are available that amplify all known species and "genotypes", yet it contains a highly variable central region at which these can be differentiated (Xiao *et al*., 1999). Secondly, there are five copies of the gene in each *Cryptosporidium* sporozoite genome, and with four sporozoites in every intact oocyst, there are twenty copies present (Le Blancq *et al*., 1997), potentially providing greater sensitivity than assays targetting single-copy genes. However, variation within multicopy genes may present an analytical challenge. Sulaiman and colleagues (1999) compared the analytical sensitivity of 11 genotyping techniques and identified that improved specificity of primer pairs and nested PCR protocols improved sensitivity over single round PCR. In an inter-laboratory comparative study of typing methods for species identification from clinical isolates, results from ssu rRNA and COWP PCR-RFLP were mutually supporting (Chalmers *et al*., 2005).

The value of typing *Cryptosporidium* isolates from routine diagnosis to the species level has been demonstrated in special studies, establishing the range of species involved in human disease, their differing epidemiology and risk factors. Eight species are known to be infectious for man: *C. parvum, C. hominis, C. meleagridis, C. felis, C. canis, C. suis, C. muris* and *C. andersoni,* although some are reported extremely rarely, particularly the latter three species, and the pathogenicity of some species for man has not been proven. In the UK, 96 % of 13,112 isolates collected since 1989 from patients with diarrhoea are either *C. parvum* or *C. hominis* (Nichols *et al*., 2006a), and other species are not restricted to immunocompromised patients (Chalmers *et al*., 2002b). In developing countries other species are more common and occur in both HIV positive and negative people (Xiao *et al*., 2001; Gatei *et al*., 2002, Cama *et al*, 2003).

Long-term molecular surveillance has shown differences in the distribution of species by person, time and place in the UK (Anon, 2002; McLauchlin *et al*., 2000; Leoni *et al*., 2006, Nichols *et al*., 2006a) and the epidemiological "noise" is reduced when typing results are incorporated into routine surveillance, as is current in Scotland (Chalmers & Pollock, 2007). Changes in the epidemiology are more readily observed and investigated (Smerdon *et al*., 2003; Hunter *et al*., 2003; Sopwith *et al*., 2005). Analytical epidemiological studies have identified specific risk factors for sporadic cases: contact with farmed animals for *C. parvum* and travel abroad, changing nappies and contact with another infected person for *C. hominis* (Hunter *et al*., 2004a) and linked species to socio-economic factors (Lake *et al*., 2007). Species identification is an important element of outbreak investigations particularly where the source

is not clear (Glaberman *et al.*, 2002), and it is important that this is underpinned by routine species identification to establish the background epidemiology. The infecting species has been identified in a large number of outbreaks worldwide, and *C. parvum* and/or *C. hominis* are always involved (Smith *et al.*, 2006; Nichols *et al.*, 2006a). This level of characterisation is also valuable in assessing public health risk when oocysts are detected in treated water, since the oocyst count may comprise species which pose a low risk to public health (Nichols *et al.*, 2006b).

Further characterisation of *Cryptosporidium* oocysts from both patients and suspected sources and vehicles of transmission is an important investigative tool during outbreaks. Not only have potential sources been better defined, for example identifying human sewage as the potential contaminant over agricultural pollution (Glaberman *et al.*, 2002) but also the relationship between source, supply and human cases and isolates circulating in the community in general (Zhou *et al.*, 2003; Cohen *et a*l., 2006; Anon 2006; Chalmers *et al.*, 2008). However, *Cryptosporidium* presents three main problems for the isolation of DNA: 1) the robust nature of the oocysts, requiring a disruption step prior to DNA extraction; 2) the richness of PCR inhibitors in the sample matrix, requiring separation during DNA extraction and/or suppression during amplification; and 3) the low number of oocysts usually present in prospectively gathered and environmental samples. Numerous methods have been used for the extraction of *Cryptosporidium* DNA, but an optimised reliable standard method is lacking and is particularly desirable for samples where small numbers of oocysts may be present (Jiang *et al.*, 2005). Appropriate PCR primer design and choice of target locus is required to avoid bias in both amplification and interpretation of results. Catchment-based studies have shown that environmental samples are likely to contain multiple species (Robinson, 2005) and repetitive PCR improves detection of these (Ruecker *et al.*, 2005). There is currently no international consensus of typing methods, and many rely on DNA sequence analysis which can be time-consuming and costly.

Not only have epidemiological differences and risk factors been identified by typing but also clinical manifestations, which vary between *C. parvum* and *C. hominis* (Hunter *et al.*, 2004b; Bushen *et al.*, 2007) and between subtypes (Cama *et al.*, 2007). Subtyping is essential to explore these and other differences in the species diversity and descriptive and analytical epidemiology which appear to vary geographically and with socio-economic groups. However, more information is required about the population genetics of *Cryptosporidium* species, which appear to be geographically linked, and distribution influenced by exposure and soci-economic factors (Mallon *et al.,* 2003; Gatei *et al.*, 2007, Hunter *et al.*, 2007, Chalmers *et al.*, 2008).

Typing methods have been compared in an initial study, the analysis of single strand conformation polymorphisms (SSCP) demonstrated that rapid methods are worth pursuing (Chalmers *et al.*, 2005). A systematic review of the literature (Robinson, 2005) has shown that none of the typing methods applied to *Cryptosporidium* have been systematically evaluated, based on principles of internationally accepted methods for evaluation of microbial typing schemes (Struelens *et al.*, 1996). Standardisation of methods and nomenclature is desirable and should be promoted.

The importance of characterisation of *Cryptosporidium* isolates to species level and of subtyping is undoubted, but future developments need to include harmonisation of rapid and more cost effective methods if these are to be adopted outside specialist laboratories and for investigation in real-time to provide information for appropriate control measures during outbreaks.

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