Virulence factor activity relationships for hepatitis E and *Cryptosporidium*

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ABSTRACT

The hepatitis E virus and *Cryptosporidium* are waterborne pathogens, each consisting of distinct taxa, genotypes and isolates that infect humans, nonhuman animal species or both. Some are associated with disease, others are not. Factors contributing to disease are extremely complicated, possibly involving differences in one or more traits associated with an organism's taxon, genotype or isolate and its infectious dose, and age or condition, as well as the host's physiology and immune status. Potential virulence factors have not yet been identified for HEV. Putative virulence factors for *Cryptosporidium* might be found in recently recognized genes involved in processes such as excystation, adherence to host cells, invasion, intracellular maintenance and host cell destruction.

Key words | cryptosporidiosis, epidemiology, hepatitis, molecular, proteins, taxonomy

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INTRODUCTION

Can we utilize our present knowledge of the hepatitis E virus or species of *Cryptosporidium* to identify or predict those species or strains likely to cause human disease? We address that question by examining the species and strains known to infect, reproduce and cause disease in humans and nonhuman animals, and identifying morphologic, biochemical and genetic characteristics potentially associated with those organisms' ability to cause disease.

HEPATITIS E VIRUS (HEV)

In terms of molecular biology and diagnostics, no specific virulence factors have been associated with any human or animal strains of HEV, although genotypes 3 and 4 generally appear less virulent than genotypes 1 and 2. At present it appears there are no specific studies to determine VFARs associated with HEV.

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HEV is an enterically transmitted human pathogen with worldwide distribution. The virus belongs to a newly described family of viruses that are small, single-stranded, positive-sense, non-enveloped RNA viruses originally placed in the Calicivirus family based on their structure and size (~30 nm). Sequence analysis of the entire genome of HEV indicated a very different genomic and replicative strategy from other viruses and it was clear it was a new family (Purcell & Emerson 2001). Genomic structure, replication strategy and comparative phylogenetic analysis has suggested the establishment of a new taxonomic family for the hepatitis e-like viruses: Family Hepeviridae, Genus Hepevirus (Emerson 2004).

The virus causes a disease that occurs sporadically as single cases as well as in large epidemics of acute hepatitis. The epidemic form occurs in areas where water supplies are contaminated with human-origin HEV. There is further

evidence of many animal species infected with a virus antigenically very similar to the human HEV, so the disease may occur as a zoonosis. A recently isolated swine virus, genetically similar to and serologically cross-reactive with human HEV, likely represents a zoonotic form of hepatitis E (Meng 2000). Some human strains appear to be readily infectious in swine and recent evidence has suggested that swine HEV (Yazaki *et al.* 2003), and also a deer strain of HEV (Tei *et al.* 2003) were transmitted to humans by consumption of raw contaminated meat. In another sporadic human case in Japan, the culprit seems to have been the family cat, which was found to have antibodies that reacted to HEV (Kuno *et al.* 2003). Interestingly, 44 of 135 cats were found to have antibodies against the virus in Japan (Okamoto *et al.* 2004). Several other animals (e.g. rodents, cattle, dogs and birds) have also been found to have anti-HEV antibodies or evidence of infection with related viruses (reviewed by Goens & Perdue (2004)). However, the vast majority of human infections worldwide appear to be from human strains.

There are several hepatitis viruses of humans that belong to several different virus families. Hepatitis A, causing the classic version of waterborne hepatitis, belongs to the *Picornaviridae*. This virus is also found worldwide and usually infects the young, yielding a measurable sero-positive adult population that in some cases makes HAV epidemics uncommon such as in some areas of Asia and Africa (Craighead 2000). Other hepatitis strains are blood-borne and widely distributed in humans but are not waterborne or enterically transmitted.

The disease caused by HEV was first recognized as a widespread occurrence of non-A, non-B hepatitis in 1980 in India (Khuroo 1980; Wong *et al.* 1980; Purcell & Emerson 2001). The virus particles were first characterized by electron microscopy and then genetic sequence analysis (Balayan *et al.* 1983; Reyes *et al.* 1990; Bradley *et al.* 1991). Ultimately the bulk of the waterborne hepatitis outbreaks in Asia and Africa were found to be caused by the hepatitis E virus (Craighead 2000; Purcell & Emerson 2001) and a direct correlation between poor sanitation and prevalence of HEV infection was made. It would appear that immunity acquired early in life from sub-clinical infections does not remain at measurable levels and consequently, even in endemically infected areas, epidemic forms of the disease can occur (Craighead 2000). In developed or industrialized countries where epidemics do not occur, anti-HEV antibody can be found in up to 7% of the population, but the source of infection inducing these antibodies is not clear (Worm *et al.* 2002).

The presence of human- or animal-origin HEV in the environment has been documented in several studies (Pina *et al.* 1998; Vaidya *et al.* 2002; Clemente-Cesares *et al.* 2003; Buti *et al.* 2004). In sewage treatment plants in Washington DC (Clemente-Cesares *et al.* 2003) investigators found viral sequences that were 91–92% identical to human HEV isolates and 98% identical to US swine viruses. Measurements of increased risks of occupational exposure to sewage in India and Switzerland showed significantly higher antibody response to HEV in the former case (Vaidya *et al.* 2003) but not the latter (Jeggli *et al.* 2004).

Hepatitis E is essentially the same disease as hepatitis A, with similar elevated liver enzyme levels. Hepatitis E should be considered if hepatitis A has been ruled out, particularly in outbreaks of waterborne hepatitis in developing countries or with patients that have recently travel to endemic areas. One of the most significant problems with HEV infections is mortality among infected pregnant women. Mortality rates range from 0.5–4% in men and non-pregnant women but can be as high as 20% in pregnant women (Hamid *et al.* 1996; Craighead 2000; Previsani & Lavanchy 2001; Purcell & Emerson 2001; Kumar *et al.* 2004). The increased mortality appears due to a severe fulminant hepatic failure (FHF) complicated by encephalopathy and disseminated intravascular coagulation (Madan *et al.* 1998; Jameel 1999; Jaiswal *et al.* 2001; Previsani & Lavanchy 2001; Khuroo & Kamili 2003).

Hepatitis E can be diagnosed using molecular tests. In the acute infection, viral sequences can be detected by PCR amplification of serum or fecal samples in approximately 50% of cases (Kurstak *et al.* 1996; Previsani & Lavanchy 2001). There is an HEV-RT-PCR test available from the CDC but it is not widely used and immuno-electron microscopy works in only about 10% of fecal samples from positive cases. (Previsani & Lavanchy 2001) Antibodies (IgM and IgG) against HEV are also specifically detected using enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) tests (Tsarev *et al.* 1993; Mast *et al.* 1998; Anderson *et al.* 1999; Innis *et al.* 2002;

Obriadina *et al.* 2002; Yu *et al.* 2003). Other commercial kits are available but not licensed in the United States. In the United States the prevalence of the disease is presently so low that molecular diagnostic 'kits' have not enjoyed widespread use beyond the research community.

In vitro propagation and production of hepatitis E virus has been reported from *in vivo*-infected primary macaque hepatocytes (Tam *et al.* 1997). Virus strains were originally isolated in human lung carcinoma cell (A549) cultures from feces of patients with acute hepatitis at Guangzhou (China) Municipal Infectious Diseases Hospital in 1993 (Wei *et al.* 1998). Subsequently the successful isolation of a sporatic G93-2 strain in A549 cultures was described with details on the etiology, molecular and biological properties, and serological relationship of this strain to other epidemic strains (Huang *et al.* 1999). This ability to propagate HEV

Table 1 | Valid species of *Cryptosporidium* and those species and genotypes identified in human infections

	USA	Peru	UK	France	Portugal	Switz.	Kenya	Thailand	Japan
Species in mammals									
C. hominis	HIV,N	HIV,C	N	HIV,N,I	HIV	HIV	HIV	HIV	HIV,N
C. parvum	HIV,N	HIV,C	N	HIV,N,I	HIV	HIV	HIV		N
C. canis	HIV	HIV,C	N						
C. felis	HIV	HIV,C	HIV,N	HIV,I	HIV	HIV		HIV	
C. muris		C?		HIV			HIV	HIV	
C. suis			N						
C. andersoni			N						
C. bovis									
C. fayeri									
C. wrairi									
C. macropodum									
C. ryanae									
C. xiaoi									
Species in birds									
C. meleagridis	HIV	HIV,C	HIV,N	HIV,I	HIV	HIV	HIV	HIV	HIV,N
C. baileyi									
C. galli									
Species in reptiles									
C. serpentis									
C. varanii									
Species in fish									
C. molnari									
C. scophthalmi									
Species in amphibians									
C. fragile									
Additional genotypes									
Pig		HIV							
Cervine	N		N						

Table compiled from data obtained from the following publications: Pieniazek *et al.* (1999), Morgan *et al.* (2000), Pedraza-Diaz *et al.* (2000, 2001*a–c*), Alves *et al.* (2001), Guyot *et al.* (2001), Xiao *et al.* (2001, 2002), Yagita *et al.* (2001), Chalmers *et al.* (2002), Gatei *et al.* (2002), Ong *et al.* (2002), Tiangtip & Jongwutiwes (2002), Palmer *et al.* (2003), Leoni *et al.* (2006), Fayer (2008) and Ryan *et al.* (2008).

strains in cell culture systems will help both in diagnosis and in facilitating vaccine research.

Since the zoonotic potential of the animal strains has been recently demonstrated in at least two cases from consumption of raw pork and venison, ingestion of any strain of HEV should be avoided. In that sense, detecting specific VFARs among the hepatitis E viruses is not important; detection of any HEV sequences would be significant. Phylogenetically, four different clades or genotypes of HEV have been characterized, all of which contain human strains (Goens & Perdue 2004). A newly characterized avian strain is substantially different structurally and phylogenetically from the human and swine strains (Huang *et al.* 2004) but its zoonotic potential is unknown. Using the 15 full-length genome sequences for HEV found in the genetic databases, conserved PCR primers can be designed to rapidly detect HEV potentially infectious for humans and thus could be used for regulatory control and further epidemiologic studies.

In summary, it appears that HEV could be transmitted from infected persons or animals through direct contact with virus in feces or fecal contamination of water, possibly through ingestion of food products from infected animals, and possibly through transfusion with contaminated blood products (although this route has not been documented). Current HEV research is focusing on genetics, diagnostics, developing vaccines for human use, on finding viruses antigenically similar to human and swine HEV in other species and determining the potential for zoonotic transmission, and on improving culture methods to aid in documenting genetically divergent isolates.

CRYPTOSPORIDIUM

Of 21 named species of *Cryptosporidium* (Table 1) and nearly 40 genotypes not identified as species or as genotypes within named species (Table 2), 8 species and 2 genotypes of *Cryptosporidium* have been detected in humans. The use of genetic analysis to identify species that have morphologically indistinguishable oocyst stages has helped to establish these taxa and has shown that *C. parvum* and *C. hominis* are the two species responsible for most human infections. Substantially fewer infections have been identified with *C. meleagridis* and even fewer infections have been reported with other species. There are also reports of infection with the pig and cervine genotypes (Xiao *et al.* 2004). The number of human infections with the cervine genotype is now greater than with all other species except *C. hominis* and *C. parvum* (Xiao & Fayer 2008). Geographic differences have been reported in prevalence attributed to these two species. Several large scale studies in the United Kingdom found that *C. parvum* was responsible for slightly more infections than *C. hominis* but a recent study in the UK found that, of 13,112 cases of cryptosporidiosis, 50.3% were associated with *C. hominis* and 45.6% were associated with *C. parvum*

Table 2 | Unique genotypes of *Cryptosporidium* based on the SSU rRNA gene sequence but not identified as species or as genotypes within named species

Mammals	Birds	Reptiles
Bear	Duck	Lizard
C. canis*,†,‡	Finch	Snake
Caribou	Goose I, II	Tortoise
Cervine I, II, III	Woodcock	
Deer		
Deer-like		
Deer mouse		
Ferret		
Fox I, II		
Horse		
Marsupial I, II		
Mongoose		
Monkey		
Mouse		
Muskrat I, II		
Opossum I, II		
Ovine§		
Pig II		
Rabbit		
Raccoon		
Seal I, II		
Sheep novel		
Skunk		
Squirrel		

Table compiled from data obtained from the following publications: Xiao *et al.* (2004), Santin *et al.* (2005) and Fayer (2008).
*Coyote genotype.
†Dog genotype.
‡Fox genotype.
§Unpublished.

(Nichols *et al.* 2006). Similarly, in other European countries, earlier studies generally showed a higher prevalence of *C. parvum* than *C. hominis* in humans whereas more recent studies have found a slightly higher prevalence of *C. hominis* than *C. parvum* (reviewed by Xiao & Fayer (2008)). Under different environmental or socioeconomic conditions, other species might be found more often.

Cryptosporidiosis has been reported in case reports or outbreaks in over 100 countries (Fayer 2008). Seroprevalence rates of 25–35% have been reported in industrialized countries but reach 42% in China, >57% in Brazil and 64% in Latin America, where water purification and sanitation standards are lower (Casemore *et al.* 1997) Seroprevalence data indicate an exposure rate of 44% among dairy farmers versus 24% in persons not associated with farming, suggesting a zoonotic link (Casemore *et al.* 1997). Farm-related outbreaks have been documented multiple times (see the review by Nichols (2008)).

Oocysts, the infective stage of *Cryptosporidium,* have been detected in groundwater, lakes, rivers, estuaries and ocean waters worldwide (reviewed by Fayer (2004)). Summarizing the findings from more than 3,700 surface water samples from 11 countries, it was found that 0 to 100% of specimens contained 0 to 252.7 oocysts per liter (Smith & Grimason 2003). Forty-five of 69 cryptosporidiosis outbreaks in North America, the UK and Japan between 1983 and 1999 were related to contaminated drinking water; the largest outbreak affected an estimated 403,000 persons (reviewed by Fayer (2004)).

The only data for which the oocyst infective dose is known for humans comes from volunteer studies in which one species of *Cryptosporidium* was tested. Oocysts of *C. parvum* from cattle from Maine, Iowa and Texas were ingested by human volunteers to compare the ID_{50} and the attack rates of isolates of the same genotype from different geographic areas (Okhuysen & Chappell 2002). The ID_{50} levels in volunteers were 1042, 87 and 9 oocysts, and the attack rates were 59, 52 and 86%, respectively, for the three isolates. Duration and severity of the disease in volunteers were milder than disease observed sporadically in the community or in outbreaks (Okhuysen & Chappell 2002). These findings indicate that factors contributing to virulence are extremely complicated, possibly involving differences in one or multiple traits such as species of *Cryptosporidium*, genotype, subgenotype, infectious dose, age or condition of the oocysts, host physiology and host immune status.

For *Cryptosporidium*, if virulence factors are considered, the processes and substances by which this parasite initiates and maintains disease in a host, these can affect the host at any time during the lifecycle from the time it enters the body until it is killed or completes the cycle and leaves.

For *Cryptosporidium* the lifecycle begins when infective sporozoites excyst from ingested oocysts and then attach to and invade host cells. It continues during a period of genetically programmed intracellular development of asexual and sexual stages involving multiplication and

Table 3 | Putative virulence factors for *Cryptosporidium*

Virulence factor genes/proteins	Putative function
Serine protease	Excystation
Aminopeptidase	Excystation
Circum-sporozoite-like glycoprotein (CSL)	Cytoadherence
Glycoprotein 900 MW (gp900)	Cytoadherence
GP 15/40/60	Cytoadherence
P23	Cytoadherence
TRAP C-1	Cytoadherence
Cp 47	Cytoadherence
CPS-500	Cytoadherence
Cp 2	Invasion, membrane integrity
Cpa135	Invasion
Secretory phospholipase A(2), (sPLA(2))	Invasion, intracellular establishment
Hemolysin H4	Membrane lysis
200 kD protein (CpABC)	Nutrient transport
CpATPase2	Biomembrane heavy metal (copper) transporter
CpATPase3	Biomembrane ion or phospholipid transporter; invasion related?
Heat Shock Protein (HSP 70)	Stress protection
Heat Shock Protein (HSP 90)	Stress protection
Type I polyketide synthase (CpPKS1)	Toxin?
Cysteine proteinase	Immune/cytokine modulation
Acetyl co synthetase	Fatty acid metabolism

Table compiled from data obtained from the following publications: LaGier *et al.* (2001, 2002), Okhuysen & Chappell (2002), Zhu *et al.* (2002), Camero *et al.* (2003), Pollok *et al.* (2003), O'Hara *et al.* (2004) and Tosini *et al.* (2004).

invasion of additional host cells. It ends with the passage of newly formed oocysts that pass out of the body in the feces. It is not known if mechanical disruption of parasitized cells, the presence of toxins or biochemical irritants, stimulation of a host's immunopathologic response by one or more antigens or immunomodulators, or combinations of these factors contribute to the virulence of a particular isolate of *Cryptosporidium*. Although the *Cryptosporidium parvum* genome has recently provided a wealth of information (Abrahamsen *et al.* 2004), little is known of the proteomics of this or other species of *Cryptosporidium*. Clues to potential virulence factors might be found in genes involved in processes such as excystation, adherence to host cells, invasion, intracellular maintenance and host cell destruction (Table 2).

Whether referred to as VFARs or SARs (structure–activity relationships), or any other associative acronym, the general premise of correlating physical (i.e. morphological) and/or molecular characteristics with human disease has been a central focus throughout most of the research involving such waterborne protozoan parasites as *Cryptosporidium*, *Cyclospora* and the Microsporidia. Because of their environmental hardiness and their emergence as potent food- and waterborne contaminants capable of rapid and widespread illness, the use of a VFAR concept initially took the form of using genetic sequence information to develop rapid and sensitive detection methodologies to discriminate between human and nonhuman pathogenic species in a variety of complex matrices. Principally, these relied on the analysis of molecular targets such as housekeeping genes (primarily the small subunit ribosomal RNA gene). In the case of *Cryptosporidium*, however, the list has expanded to include a number other identifiable target genes. In addition, genes have been identified that relate to a variety of biological processes required for the establishment and maintenance of the parasite in the host, as cited in Table 3. As such, the expanding molecular database for *Cryptosporidium* is becoming the foundation for bioinformatics, genomics and functional genomics, and hence an increasingly correlative tool to predict human pathogenicity.

Virulence factors, virulence and risk are intertwining elements that go beyond the mere correlation between the presence or absence of putative virulence factor isoforms and human pathogenicity. Risk, and hence virulence, can be seen as a compilation of many factors pertaining not only to the pathogen (a particular human genotype or species of *Cryptosporidium*) but the host (individual or population subset). Therefore, virulence factors such as infectious dose, invasion receptor densities, or protease activities and the host condition (e.g. immunocompromised or immunosuppressed individuals) may all influence the level of pathogenicity, i.e. the severity and duration of the resulting illness for any one individual.

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