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Explaining unexplained diarrhea and associating risks and infections

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Abstract

Gastrointestinal illnesses are common afflictions. However, knowledge of their etiology is often lacking. Moreover, most cases of infections with reportable enteric pathogens (*Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella*, *Shigella*, *Yersinia*, *Cryptosporidia* and *Giardia*) have sporadic modes of acquisition, yet control measures are often biased towards mitigation of risks discerned by outbreak analysis. To determine the etiology of unexplained diarrhea it is important to study populations that can be matched to appropriate controls and to couple thorough classic microbiologic evaluation on receipt of specimens with archiving and outgrowth capabilities. Research evaluations should address the potential roles of a broad panel of candidate bacterial pathogens including diarrheagenic *E. coli*, *Listeria monocytogenes*, Helicobacters and *jejuni* Campylobacters, and also apply novel massively parallel sequencing and nucleic acid detection technologies that allow the detection of viral pathogens. To fill voids in our knowledge regarding sources of known enteric pathogens it will be critical to extend case-control studies to assess risk factors and exposures to patients with non-epidemic illnesses and to appropriate controls. By filling these gaps in our knowledge it should be possible to formulate rational prevention mechanisms for human gastrointestinal illnesses.

Keywords: diarrhea/epidemiology, diarrhea/microbiology, risk factors, animals, domestic

Introduction

Our abilities to prevent gastrointestinal illnesses transmitted via water, food and animal vehicles are dependent on two critical assumptions: first, that we know the causative agents of these illnesses and, second, that we know the risk factors for acquiring infections with these known agents. Unfortunately proposed risk factors for known enteric infections are primarily based on data from outbreaks despite the fact that the vast majority of these infections are sporadic and not outbreak-related. Furthermore, the etiologies of many gastrointestinal illnesses are not known. Even many outbreaks of gastrointestinal illnesses cannot be attributed to a specific agent. Here we outline gaps in our knowledge so as to prompt investigation into discerning the causes of unexplained enteric illnesses and to determining the mechanism of acquisition

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Table 1. 2005 Washington State incidence rates/100,000 population by organism and county (Washington State Department of Health, 2006)

	King	Whatcom	Yakima	Washington State incidence rate (number)
Campylobacter	18.6	36.5	50.6	16.7 (1045)
Cryptosporidium	3.0	*(1 case)	3.1	1.5 (94)
Cyclospora	n/a	n/a	n/a	0.1 (5)
E. coli O157:H7	2.4	5.0	1.8	2.4 (149)
Giardia	7.7	8.8	12.2	7.0 (437)
Salmonella	11.8	8.8	22.7	10.0 (626)
Shigella	4.0	2.8	12.6	3.0 (185)
Yersinia	0.6	*(2 cases)	*(1 case)	0.3 (19)

*Incidence rates not calculated in the report for <5 cases.

of sporadic infections with known reportable enteric pathogens. It was not possible to consider critically each candidate pathogen, each disorder and all studies. However, we hope that this review increases the appreciation that there are large voids in our knowledge about what causes human enteric infections and about how sporadic human infections from known pathogens are really acquired and that these voids need to be filled to increase food microbiologic safety.

The undetermined sources of reportable enteric infections (REIs)

Food-borne exposures continue to contribute to burden of disease but geographic trends also suggest environmental risks

The sources of most gastrointestinal infections remain unknown despite popular and medical misconceptions based on outbreak data that certain vehicles are the predominant modes of transmission of certain pathogens. Indeed when intense investigations are applied it has become apparent that vehicles that have caused well-publicized epidemics are probably not responsible for many sporadic infections.

For example, during calendar year 2005 there were 2461 total cases of infection caused by *Campylobacter*, *Salmonella*, *Giardia*, *Escherichia coli* O157, *Shigella* and *Yersinia* in the State of Washington (Washington State Department of Health, 2006). However, of these reported infections only 2.4% overall were attributed to food-borne outbreaks (ranging from 0% for *Giardia* to 17% for *E. coli* O157:H7). Based on 1998–2002 data (Lynch *et al.*, 2006) only 0.8, 7.8, 5.3, 0.3, 30.9 and 1.6% of the reported infections caused by *Campylobacter*, *Salmonella*, *Shigella*, *Cryptosporidium*, *E. coli* O157 and *Yersinia* respectively could be attributed to food-borne outbreaks. Extrapolating from 2001 data (Blackburn *et al.*, 2004; Yoder *et al.*, 2004) only 0.6 and 0.03% of these infections are secondary to recreation and drinking water outbreaks respectively. Clearly the majority of REIs appears sporadic and while these illnesses might be associated with occult epidemics their sources remain undetermined.

Enteric infections account for an estimated 76 million illnesses 325,000 hospitalizations and 5000 deaths in the US annually (Mead *et al.*, 1999). The USDA Economic Research Service estimates that costs related to five food-borne pathogens (*Campylobacter*, *Salmonella*, *E. coli* O157, non-O157:H7 Shiga toxin-producing enterohemorrhagic *E. coli*, and *Listeria monocytogenes*) account for US $6.9 billion annually (http://www.ers.usda.gov/briefing/Food-borneDisease/features.htm). These infections are often referred to as food-borne and yet the epidemiology and scientific understanding of such enteric illnesses are evolving. For example, new pathogens have emerged (e.g. *E. coli* O157) and many organisms have reservoirs in healthy animals from which spread can occur directly or through an increasing variety of food. In addition changes in US food production practices and the increased availability of imported foodstuffs play increasing roles in disease transmission as do recreational and drinking water vehicles (Mead *et al.*, 1999). Therefore we need thorough ascertainment of evolving risk factors related to infection and it is especially critical to study sporadic cases because of their numeric predominance. An enhanced understanding of the precise nature of these infections will inform effective and economically efficient prevention strategies.

One such area and risk factor that needs investigation is the environment. Accumulated data suggest that residents of rural locales especially those with high frequencies of exposure to livestock are at increased risk of acquiring sporadic enteric infections (Haus-Cheymol *et al.*, 2006; Strachan *et al.*, 2006). Within Washington State for example Yakima and Whatcom counties have the largest and second largest number of cattle farms and the highest and third highest densities of cattle respectively (United States Department of Agriculture, 2002); these counties also have the State's highest incidence of *E. coli* O157:H7 infections (Washington State Department of Health, 2006) (Table 1). Other studies demonstrate the importance of animal contact with acquisition of *Campylobacter* and *E. coli* O157:H7 infections (Innocent *et al.*, 2005;

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Marcus *et al.*, 2007) and seroepidemiologic data and other data support the concept of increased risk of *E. coli* O157:H7 in rural residents (Michel *et al.*, 1999; Evans *et al.*, 2000; Valcour *et al.*, 2002; Haack *et al.*, 2003; Carroll *et al.*, 2005). In one attempt to find vehicles for sporadic enteric infections among Danish children Ethelberg *et al.* (2006) associated day care attendance and contact with other individuals with diarrheal illness as risk factors. However, after eliminating viral illnesses and diarrhea of unknown etiology, these investigators found an association between foreign travel and socioeconomic factors only. Domestic vehicles of acquisition were not identified.

Below we exemplify four pathogens, discuss their sporadic and geographic epidemiologies, and highlight the need to determine more about modes of acquisition of these infecting organisms especially non-food sources.

The case of *E. coli* O157:H7 infections

While large outbreaks implicated ground beef as a vehicle of *E. coli* O157:H7 infection (Riley *et al.*, 1983; Bell *et al.*, 1994) most *E. coli* O157:H7 infections are not attributable to identifiable outbreaks. Additionally, when ground beef is included in surveys of sporadic disease this vehicle does not explain the vast majority of cases (Michel *et al.*, 1998; Evans *et al.*, 2000; Marcus *et al.*, 2007). Subsequent outbreaks have suggested a considerably wider range of potential vehicles such as alfalfa (Breuer *et al.*, 2001; Ferguson *et al.*, 2005), radish sprouts (Michino *et al.*, 1999), lettuce (Ackers *et al.*, 1998), unpasteurized juices (Cody *et al.*, 1999), water (Swerdlow *et al.*, 1992; Krewski *et al.*, 2002; Olsen *et al.*, 2002), unpasteurized dairy products (Djuretic *et al.*, 1997; Honish *et al.*, 2005) and spinach (Centers for Disease Control and Prevention, 2006). Additionally, there is discordance between case reporting and case occurrence which could lead to incomplete epidemiologic assessments of outbreaks (Michel *et al.*, 2000).

Sporadic and outbreak-related data suggest environmental and agricultural sources for infection via contact with animals and their excreta (Parry *et al.*, 1998; Locking *et al.*, 2001; Innocent *et al.*, 2005). Interestingly *E. coli* O157:H7 has a disproportionately higher incidence in the rural UK (Evans *et al.*, 2000) and rural residents have serologic evidence for increased exposure to this pathogen in the US (Haack *et al.*, 2003), Canada (Reymond *et al.*, 1996) and Scotland (Strachan *et al.*, 2006). These epidemiologic findings are consistent with data demonstrating cattle as an important reservoir of *E. coli* O157 and the ability of the organism to survive in the environment for months (Wang *et al.*, 1996; Besser *et al.*, 1997; Rahn *et al.*, 1997; Kudva *et al.*, 1998). In one multistate study several factors were identified with largely sporadic cases of *E. coli* O157:H7 infection: eating rare hamburgers, imbibing untreated surface water and contact with cattle, whereas produce consumption in general diminished risk (Marcus *et al.*, 2007).

The case of *Salmonella* infections

Salmonella infections could be acquired by exposure to agricultural reservoirs. *Salmonella* has been documented in swine and dairy farms and outbreak data have demonstrated human infection following farm animal contact (Maguire *et al.*, 1993). However, epidemiologic data have not demonstrated such strong rural associations in North America. Foods certainly transmit Salmonella and outbreak vehicles have included sprouts eggs (St Louis *et al.*, 1988; Sobel *et al.*, 2000; Kimura *et al.*, 2004; Chittick *et al.*, 2006), poultry (Chittick *et al.*, 2006), milk (De Buyser *et al.*, 2001), orange juice (Cook *et al.*, 1998; Public Health Agency of Canada, 1999; Centers for Disease Control and Prevention, 1991), beef (Centers for Disease Control and Prevention, 1981; Davies *et al.*, 1996; Wegener and Baggesen, 1996), fresh produce (Centers for Disease Control and Prevention, 1991; Sivapalasingam *et al.*, 2004) including pre-bagged ready-to-eat salads (Sagoo *et al.*, 2003), cilantro (Campbell *et al.*, 2001), and peanut butter (Scheil *et al.*, 1998; Anon., 2007). Egg (Hennessy *et al.*, 2004; Marcus *et al.*, 2007) and poultry (Humphrey *et al.*, 1988; Kimura *et al.*, 2004) consumption and exposure to birds and reptiles (Marcus *et al.*, 2007) have been identified as risks for sporadic infections acquired domestically in the United States. Interestingly 0.2% of retail ready-to-eat salads in the UK (Sagoo *et al.*, 2003) and 7% of Seattle retail sprouts samples (Samadpour *et al.*, 2006) were contaminated with *Salmonella*. Less information is available on the sources of sporadic infection with *Salmonella*. Limited data suggest that consumption of chicken particularly commercially prepared chicken, home-prepared eggs (Kimura *et al.*, 2004; Marcus *et al.*, 2007), shrimp and pork and eating sprouts in general or even having sprouts in the household are associated with salmonellosis (Denno *et al.*, 2007). Exposure to pets particularly reptiles and amphibians and poultry have also been implicated as has recreational beach play (Mermin *et al.*, 2004; Jones *et al.*, 2006; Denno *et al.*, 2007).

The case of *Campylobacter* infections

Campylobacter, the most common REI in many locales (Blaser *et al.*, 1982; de Wit *et al.*, 2001a, b; Denno *et al.*, 2005; Centers for Disease Control and Prevention, 2006; Washington State Department of Health, 2006), has been detected in retail meats (Nielsen *et al.*, 1997; Zhao *et al.*, 2001; Jorgensen *et al.*, 2002; Fitch *et al.*, 2005) and is transmitted via food particularly poultry (Hopkins *et al.*, 1984; Oosterom *et al.*, 1984; Deming *et al.*, 1987; Kapperud *et al.*, 1992, 2003; Ikram *et al.*, 1994; Schorr *et al.*, 1994; Adak *et al.*, 1995; Eberhart-Phillips *et al.*, 1997; Studahl and Andersson, 2000; Effler *et al.*, 2001; Rodrigues *et al.*, 2001; Wingstrand *et al.*, 2006) including poultry brought into the home raw (Kapperud *et al.*, 1992; Ikram

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et al., 1994). Transmission via recreational (Schonberg-Norio *et al.*, 2004) and contaminated water (Hopkins *et al.*, 1984; Kapperud *et al.*, 1992, 2003; Adak *et al.*, 1995; Eberhart-Phillips *et al.*, 1997; Schonberg-Norio *et al.*, 2004; O'Reilly *et al.*, 2007) has been observed primarily during outbreaks. However, the relative risk and specific nature of these risks (i.e. via consuming any versus consuming undercooked food commercially prepared versus home-cooked or via cross-contaminated food) have varied.

Consumption of unpasteurized milk (Hopkins *et al.*, 1984; Anon., 1986; Schmid *et al.*, 1987; Birkhead *et al.*, 1988; Studahl and Andersson, 2000; Potter *et al.*, 2003) is a risk factor for sporadic *Campylobacter* infections. There are higher rates of *Campylobacter* infection in rural populations (Thompson *et al.*, 1986) suggesting agricultural links. Contact with animals (including poultry cattle swine cats and dogs) that excrete *Campylobacter* are likely sources of human infection (Blaser and Reller, 1981; Hopkins *et al.*, 1984; Deming *et al.*, 1987; Salfield and Pugh, 1987; Kapperud *et al.*, 1992, 2003; Adak *et al.*, 1995; Eberhart-Phillips *et al.*, 1997; Studahl and Andersson, 2000; Tenkate and Stafford, 2001). Eating at a table service restaurant (Friedman *et al.*, 2004) and contact with persons with diarrhea (Oosterom *et al.*, 1984) are other likely vehicles.

The case of *Shigella* infections

Humans and several other primates are the only natural hosts for *Shigella*. However, *Shigella* has been responsible for numerous food-borne outbreaks (Keusch and Bennish, 1998) probably because of human contamination of food. Incidence rates are higher in economically disadvantaged minorities including Hispanics (Keusch and Bennish, 1998). Risk factors for sporadic cases of *Shigella* infection are however, unknown, in general populations.

The preponderance of data regarding routes of transmission of these four REIs arises from outbreak investigations and yet the majority of infections are sporadic. The identification of vehicles of acquisition for specific pathogens contributes to overall knowledge regarding risk factors for infection. This information correlated with regional variations related to sources can provide an evidence base to guide hazard control and other prevention efforts.

The undetermined cause of many cases of enteric infections

The infectious causes of most cases of diarrhea remain unknown even with intense microbiologic evaluation. A roster of plausible candidate pathogens is now available but it is critical to determine if such agents are found more frequently in cases than controls. Measures to advance food safety using epidemiologic science require a thorough knowledge of the causes of human diseases. Our ignorance of the cause of many cases of gastroenteritis is a major impediment to their prevention. Genomic sequencing and nucleic acid arrays now permit novel powerful and minimally biased analyses of human specimens.

The elusive cause of diarrhea

Even when broad efforts are applied a bona fide microbiologic cause of diarrhea frequently cannot be established (Caeiro *et al.*, 1999; Waters *et al.*, 2000; de Wit *et al.*,2001a, b; Cohen *et al.*, 2005; Denno *et al.*, 2005, 2007; Klein *et al.*, 2006; Vernacchio *et al.*, 2006a, b). For the purposes of this review 'bona fide' diarrheagenic agents found to varying extents in North America (many of which are acquired from contaminated food or water) are *Campylobacter jejuni*, *E. coli* O157:H7, selected non-O157:H7 Shiga toxin-producing *E. coli* (STEC), *Salmonella*, *Shigella*, *Vibrio* and *Yersinia* spp.; *Clostridium difficile* following antibiotic administration in non-infants; *Giardia lamblia*; *Entamoeba histolytica*, *Cryptosporidium parvum*, *Microsporidia*, *Cyclospora*, *Isospora*; rotavirus caliciviruses, astroviruses and adenoviruses. Diverse factors underlie our ignorance about the causes of specific cases of diarrhea (Sherman and Tarr, 2005) including expense (thorough evaluations cost hundreds of dollars) and futility (diagnosis rarely leads to treatment or when pathogens are detected late in illness the offending agent is often already cleared or the patient is already improved). Importantly the currently available methods for the detection of many of these pathogens lack sensitivity and in some cases specificity. Even in outbreaks which offer unique chances to determine causes, etiologies often escape detection. For example, in 2004 29 of Washington State 58 enteric infection clusters were classified as caused by an unknown agent or characterized as 'viral' without an agent being recovered (http://www.doh.wa.gov/notify/nc/incidence/food-borne.pdf).

Candidate pathogens warrant assessment as to their association with human disease in the United States

The problem of determining etiologies in cases of gastroenteritis is magnified by the challenges of defining exactly what constitutes a pathogen. Many non-O157:H7 STEC (Tarr and Neill, 1996; Johnson *et al.*, 2006), non-*jejuni Campylobacter* (Lawson *et al.*, 1999), *E. coli* with enterotoxigenic (ETEC), enteropathogenic (EPEC) or enteroaggregative (EAggEC) loci (Nataro and Kaper, 1998) *L. monocytogenes* (Riedo *et al.*, 1994; Dalton

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et al., 1997; Miettinen *et al.*, 1999; Aureli *et al.*, 2000; Ooi and Lorber, 2005), *Aeromonas* and *Pleisiomonas* spp. (Essers *et al.*, 2000; Shigematsu *et al.*, 2000), *Helicobacter pylori* (Frenck and Clemens, 2003), *Blastocystis hominis* (Cirioni *et al.*, 1999), *Dientamoeba fragilis* (Cuffari *et al.*, 1998; Preiss *et al.*, 1991), and community-acquired non-antibiotic-associated *C. difficile* (Riley *et al.*, 1995) can be excreted in the absence of diarrhea (Cobb *et al.*, 1996; Hellard *et al.*, 2000; Svenungsson *et al.*, 2000) calling into question their pathogenicity. Additionally, many such agents are 'nominated' as pathogens only because they possess a single virulence trait. In reality, the ability to cause disease in humans probably depends on additional virulence loci. Non-O157:H7 STEC are good examples of this challenge. For example, merely possessing genes encoding Shiga toxin does not by itself define a human pathogen (Tarr and Neill, 1996) because auxiliary bacterial virulence traits are generally necessary for disease association (Beutin *et al.*, 2004; Bielaszewska *et al.*, 2005, 2007). Nonetheless, some non-O157:H7 STEC are clearly pathogens by virtue of their identification in clusters of disease (Paton *et al.*, 1996, 1999; Hashimoto *et al.*, 1999; Feng *et al.*, 2001; McCarthy *et al.*, 2001; Werber *et al.*, 2002; Misselwitz *et al.*, 2003; Brooks *et al.*, 2004; Bielaszewska *et al.*, 2006).

Candidate diarrheagenic (for humans) bacteria, such as non-O157:H7 Shiga toxin-producing *E. coli*, are found in food (Samadpour *et al.*, 1994, 2006; Acheson, 2000; Jaeger and Acheson, 2000) and food animals (Gyles *et al.*, 1998; Hussein and Bollinger, 2005; Keller *et al.*, 2007; Mora *et al.*, 2007). Confirming or refuting the role of these agents as pathogens is necessary to prioritize research on the ecology, biology and reservoirs of such organisms. Rigorous data form the foundation for appropriate regulations. Filling this void in our knowledge will lead to outcome measures for strategies to target microbial contamination of food by identifying or refuting pathogenicity of organisms that might be transmitted by food.

Technology now permits accurate and economical non-culture detection of pathogens

Current strategies for identifying an infectious agent in cases of gastroenteritis employ cumbersome Balkanized slow and expensive technology. For example, identification of bacterial etiologies relies on the performance of cultures on variably selective agar and broth media and then characterizing resulting colonies or broth cultures using serologic, biochemical and other phenotypic assays (such as toxin detection). Antigen detection or toxin assays applied directly to stool or stool filtrate are employed to detect rotavirus adenovirus astrovirus and *C. difficile*. Detection of parasites relies on direct observation of ova or organisms or detection of parasitic antigens. Moreover, these technologies have been developed to detect organisms based on known characteristics and thus are blind to new or evolving pathogens. However, emerging technologies may hold promise for the revolution of diagnostic methods and strategies and for the identification of emerging agents of disease.

Novel viral pathogens

The spectrum of human diarrheagenic viruses is unknown but probably broad. Traditional technologies for viral identification even if utilizing molecular-genetic detection rely on viral cultivation immune electron microscopy (Kapikian *et al.*, 1972), animal inoculation and serologic–epidemiologic studies to discover or validate new pathogens. These efforts are potentially applicable to situations where serum or fresh stool is available and there is a good candidate agent. However, in view of their intensity and cost, these techniques are best applied either to clusters of infection or to high frequency agents of disease. The effort involved in identifying antibody responses, viral cultures or animal challenges to find novel agents would be exorbitant if the percent of infected patients is low.

However, after a candidate viral etiologic agent is identified and its genome is sequenced, individualized assays such as enzyme immunoassay (EIA) and genetic detection methodologies, in particular the polymerase chain reaction (PCR), can be used. These techniques however, have limitations. EIA cannot discover novel agents and is critically dependent on the quality of antiserum/antibody employed. EIAs are additionally constrained by existing knowledge such that antibodies can only be made to antigens after the identities of the expressed molecules are known. PCR is sensitive but its use is predicated upon sufficient knowledge to design primers. Also, while PCR that detects multiple different loci simultaneously (multiplex PCR) does enable the identification of more than one agent in a single analyte, it is often difficult to design compatible primer sets for multiplexing without considerable empiric validation. Also, one is limited to a relatively small number of targets in a single assay and identification using degenerate PCR (allowing variation in target) requires confirmatory efforts such as sequencing hybridization of the resulting amplicons or restriction analysis. Clearly, novel approaches that circumvent technologic difficulties of viral detection and the biases inherent in EIAs and PCR are needed to discover novel human viruses. Viral microarrays are one technologic advance to discover novel viruses associated with food-borne illness.

A validated DNA microarray for highly parallel viral detection eliminates many theoretical and practical problems of viral discovery and diagnosis. Initially >140 viral genome sequences were used to design a long (70-mer) DNA microarray on glass slides (Eisen and Brown, 1999) to capture diverse viral nucleic acids from polymicrobial milieus (Wang *et al.*, 2002). The most

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conserved 70-mers were selected from each known virus to maximize the probability of detecting all members of a given family including unsequenced novel or variant members. This approach successfully detected a panel of known human respiratory viruses *in vitro* and nasal washings from experimentally and naturally infected subjects (Wang *et al.*, 2002). These results were independently confirmed by conventional virus-specific PCR assays.

A second generation array incorporating oligonucleotide elements from every fully sequenced reference genome (~1000 viruses) was subsequently developed and validated when severe acute respiratory syndrome coronavirus (SARS) appeared in 2003 (Wang *et al.*, 2003). Specimen DNA that binds to the array can be scraped and sequenced (Wang *et al.*, 2003).

The value of screening human specimens with this highly parallel microarray assay is evident. In addition to screening for all known diarrheagenic viruses this approach provides a rational basis and strategy for virus discovery. Unrecognized members of existing viral families can be detected by hybridization to the highly conserved sequences represented on the microarray. Moreover, this system's use of 70-mers can tolerate significant mutations and still yield detectable hybridization. This technique permits the parsimonious application of valuable specimens because the nucleic acids are derived only from two different extraction processes: one for RNA and one for DNA. Microarray analysis is less economical than multiplex PCR but the multitude of agents that can be found is dramatically larger.

A complementary approach is the use of in-depth massively parallel sequencing. This approach has been applied to respiratory secretions from patients with respiratory tract infections to identify human bocavirus (Allander *et al.*, 2007b) and two novel polyomaviruses from humans (Allander *et al.*, 2007a; Gaynor *et al.*, 2007). Briefly, secretions from patients are cloned (with or without pooling) and sequenced. Novel sequences identified from the sequenced libraries that appear microbial in origin are then used to create primers to interrogate specimens from patients and controls with conventional PCR to assess potential associations with disease. Mass sequencing of stool samples from healthy individuals has revealed the presence of a large diversity of phage and plant viruses (Breitbart *et al.*, 2003; Zhang *et al.*, 2006).

Novel bacterial pathogens

Classic bacteriology will not identify many agents that are postulated to cause diarrhea. Examples include ETEC which have been isolated in this country (Caeiro *et al.*, 1999; Beatty, 2004) and EaggEC (Nataro, 2005) which were recently associated with diarrhea in Cincinnati children (Cohen *et al.*, 2005). Classic microbiologic studies would also overlook *H. pylori* which has been proposed to cause infectious acute childhood diarrhea (Sullivan *et al.*, 1990; Passaro *et al.*, 2001) though there is contradictory literature on the diarrheagenic role of this agent (Kehrt *et al.*, 1997; Rahman *et al.*, 1998; Rothenbacher *et al.*, 2000) and it is somewhat unlikely that this gastric pathogen has a major role in diarrhea. *L. monocytogenes*, which has been incriminated in epidemic gastroenteritis in the United States (Riedo *et al.*, 1994; Dalton *et al.*, 1997) and overseas (Miettinen *et al.*, 1999; Aureli *et al.*, 2000) though not in sporadic cases of diarrhea in at least one North American study (Sauders *et al.*, 2005) would also be overlooked. The epsilon-proteobacteria encompasses two families: the Campylobacteraceae and the Helicobacteriaceae which have additional members that might be unrecognized as causes of diarrhea (Fox, 2002). *C. jejuni* and *Campylobacter coli* are most commonly recognized as a cause of acute gastroenteritis but other pathogenic epsilon-proteobacteria *Campylobacter upsaliensis*, *Campylobacter hyointestinalis*, *Campylobacter fetus* and *Arcobacter* spp. (Lawson *et al.*, 1999; Lehner *et al.*, 2005) have been associated with acute gastroenteritis and are potential food-borne pathogens. *Arcobacter* spp. are potentially important emerging food-borne pathogens that potentially share transmission vehicles similar to those that transmit *C. jejuni* (Son *et al.*, 2007). A number of non-*pylori* intestinal Helicobacters have been suggested as causes of human diarrhea (Fox *et al.*, 2000; Solnick and Schauer, 2001). *Helicobacter canadensis* (Fox, 2002) and *Helicobacter pullorum* (Melito *et al.*, 2000) have been reported to cause acute gastroenteritis resembling that caused by *C. jejuni* and *C. coli*, while *Helicobacter fennelliae* and *Helicobacter cinaedi* appear to have a predilection for causing gastroenteritis and invasive disease in immunocompromised patients (Andersen, 2001). The recognition of these organisms as pathogens has been hampered by the fact that the routine microbiologic techniques developed to isolate *C. jejuni* and *C. coli* are suboptimal for the growth of these atypical organisms (Lastovica and le Roux, 2000).

We have recently published two studies that demonstrate the high proportion of diarrheal illness that remains etiologically unexplained despite intensive microbiologic investigation applied to specimens. The studies were performed at the Seattle Children's Hospital and Regional Medical Center Emergency Department (Klein *et al.*, 2006) and two additional urban ambulatory practices in Seattle (Denno *et al.*, 2005). These sites serve a geographically and demographically diverse pediatric outpatient population. All stools were tested for *Aeromonas* spp., *C. jejuni*, *E. coli* O157:H7, non-O157:H7 STEC *Pleisiomonas shigelloides* and *Salmonellae*, *Shigella*, *Vibrio* and *Yersinia* spp. Stools submitted in sufficient quantity were additionally tested for parasites by microscopy (wet mount, concentrate, trichrome and auramine stains, and fluorescent antibody analysis for *G. lamblia* and *C. parvum*). *C. difficile* toxin by cytotoxicity assay

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and adenovirus, astrovirus, and rotavirus by EIA. The resulting data indicate that many cases of diarrhea even in high acuity populations such as a children's hospital emergency department remain unexplained in terms of etiology even though a broad and expensive array of microbiologic testing is performed. Even though most cases of diarrhea currently remain unexplained, a subset is plausibly caused by novel viruses *E. coli*, *L. monocytogenes* or epsilon-proteobacteria and this area is worthy of further and intense investigation.

Clearly, thoroughly evaluating stools for known and yet-to-be discovered viruses and candidate bacterial pathogens that would be overlooked on standard cultures is warranted. Knowing the causes of human gastrointestinal infections is inherent to food safety and currently our knowledge is limited. Data regarding disease etiology are needed to assess risk, improve product safety and prevent disease. By applying advanced technology to associate unexplained diarrhea in case-control studies with known and unknown agents, investigators will be able to establish or refute the role of certain organisms in human disease. The establishment of an agent as a pathogen will lead to interventions to reduce its ability to infect humans. Such data are needed to provide guidance on practices aimed at controlling hazards assessing risks and directing resources.

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