

Guidelines for drinking-water quality

SECOND EDITION



Addendum
**Microbiological agents
in drinking water**



World Health Organization
Geneva

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Guidelines for drinking-water quality

SECOND EDITION

Addendum ***Microbiological agents in drinking water***



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Preface

Between 1993 and 1997 the World Health Organization (WHO) published the second edition of *Guidelines for drinking-water quality* in three volumes: Volume 1, *Recommendations*, in 1993, Volume 2, *Health criteria and other supporting information*, in 1996, and Volume 3, *Surveillance and control of community supplies*, in 1997. Development of the *Guidelines* in this second edition was organized and carried out jointly by WHO headquarters and the WHO Regional Office for Europe.

At the Final Task Group Meeting (Geneva, Switzerland, 21–25 September 1992), when the second edition of the *Guidelines* was approved, it was agreed to establish a continuing process of updating, with a number of chemical substances and microbiological agents subject to periodic evaluation. Addenda containing these evaluations will be issued as necessary until the third edition of the *Guidelines* is published, approximately 10 years after the second edition.

In 1995, a Coordinating Committee for the Updating of the WHO *Guidelines for drinking-water quality* agreed on the framework for the updating process and established three working groups to support the development of addenda and monographs on chemical aspects, microbiological aspects, and protection and control of water quality. The Committee selected microbiological agents for review, and identified lead individuals and institutions for the preparation of microbiological review documents and support individuals and institutions to assist in their review and finalization. Institutions and individuals from Australia, Austria, Canada, France, Germany, India, Indonesia, Italy, Japan, Netherlands, South Africa, Thailand, United Kingdom, and USA were involved in the preparation of the documents.

The draft microbiology review documents were submitted to a number of scientific institutions and selected experts for peer review. Comments were taken into consideration before the documents were submitted for final evaluation by the 1998 meeting of the Working Group on Aspects of Protection and Control and of Microbiological Quality.

The microbiological review documents contained in this addendum supersede those previously published in Volumes 1 and 2 of the second edition of the *Guidelines for drinking-water quality*. Their more extensive coverage of individual pathogens reflects the need for more substantial review information to assist

and support the further development of the *Guidelines*, particularly with respect to microbiological aspects.

The reviews do not conclude with the definition of “safe” or “tolerable” exposures as is the case with the analogous chemical reviews in the *Guidelines*. Microbiological quality may vary rapidly and widely and the health consequences of short-term exposures are typically significant. These features, combined with the incompleteness of current knowledge regarding the identity of waterborne pathogens and the poor availability and speed of analytical techniques for recognized pathogens, mean that defining safe exposures and monitoring their achievement are not generally the preferred means of control. Emphasis is therefore placed upon understanding conditions likely to ensure the safety of drinking-water supplies and monitoring their fulfilment more directly. The microbiology reviews published here therefore summarize current knowledge regarding quantitative aspects of transmission, attenuation, and removal of individual pathogens, and regarding the effectiveness of measures for the interruption of transmission.

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Individual contributions to the content of this addendum are acknowledged on the first page of each main section of the text.

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Aeromonas¹

Description

Species of *Aeromonas* are Gram-negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that occur ubiquitously and autochthonously in aquatic environments. Although historically the *Aeromonas* genus has been placed in the family Vibrionaceae (Popoff, 1984), there have been proposals to place it in its own family, the Aeromonadaceae (Colwell, MacDonnell & De Ley, 1986). The aeromonads share many biochemical characteristics with members of the Enterobacteriaceae, from which they are primarily differentiated by being oxidase-positive. The genus includes at least 13 genospecies, among which are the mesophilic *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. schubertii*, and the non-motile, psychrophilic *A. salmonicida*.

A. salmonicida is a fish pathogen and has not been associated with human infection. By contrast, the mesophilic species have been associated with a wide range of infections in humans (Janda & Abbott, 1996). Although members of the genus have classically been divided into three biochemically differentiated groups (typified by *A. hydrophila*, *A. caviae*, and *A. sobria*), these contain a number of genospecies, to which new species have been added (Carnahan & Altwegg, 1996). Currently the genus is made up of 17 DNA hybridization groups representing a range of genospecies and phenospecies (see Table 1).

The mesophilic aeromonads have been commonly isolated from patients with gastroenteritis although their role in disease causation remains unclear. They are also associated with sepsis and wounds, and with eye, respiratory tract, and other systemic infections (Janda & Duffey, 1988; Janda & Abbott, 1996; Nichols et al., 1996); see Table 2. Many of the systemic infections arise following contamination of lacerations and fractures with *Aeromonas*-rich waters.

The species principally associated with gastroenteritis are *A. caviae*, *A. hydrophila*, and *A. veronii* biovar *sobria* (Joseph, 1996); *A. caviae* is particularly associated with young children (under 3 years of age). Many studies have resulted

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Table 1. Genospecies and phenospecies of the genus *Aeromonas*^a

DNA hybridization group	Reference strain (T = type strain)	Genospecies	Phenospecies
1	ATCC 7966 ^T	<i>A. hydrophila</i>	<i>A. hydrophila</i>
2	ATCC 51108 ^T	<i>A. bestiarum</i>	<i>A. hydrophila</i>
3	ATCC 33658 ^T	<i>A. salmonicida</i>	<i>A. salmonicida</i>
3	CDC 0434-84	<i>A. salmonicida</i>	<i>A. hydrophila</i>
4	ATCC 15468 ^T	<i>A. caviae</i>	<i>A. caviae</i>
5A	CDC 0862-83	<i>A. media</i>	<i>A. caviae</i>
5B	CDC 0435-84	<i>A. media</i>	<i>A. media</i>
6	ATCC 23309 ^T	<i>A. eucrenophila</i>	<i>A. eucrenophila</i>
7	CIP 7433 ^T	<i>A. sobria</i>	<i>A. sobria</i>
8	ATCC 9071	<i>A. veronii</i>	<i>A. veronii</i> biovar <i>sobria</i>
9	ATCC 49568 ^T	<i>A. jandaei</i>	<i>A. jandaei</i>
10	ATCC 35624 ^T	<i>A. veronii</i>	<i>A. veronii</i>
11	ATCC 35941	Unnamed	<i>Aeromonas</i> sp (ornithine-positive)
12	ATCC 43700 ^T	<i>A. schubertii</i>	<i>A. schubertii</i>
13	ATCC 43946	Unnamed	<i>Aeromonas</i> Group 501
14	ATCC 49657 ^T	<i>A. trota</i>	<i>A. trota</i>
15	CECT 4199 ^T	<i>A. allosaccharophila</i> ^b	<i>A. allosaccharophila</i> ^b
16	CECT 4342 ^T	<i>A. encheleia</i> ^b	<i>A. encheleia</i> ^b

^a Modified from Carnahan & Altwegg, 1996

^b The taxonomic status of *A. allosaccharophila* and *A. encheleia* remains to be confirmed. A further new species, *A. popoffi* (unassigned DNA hybridization group), has also been proposed

in the isolation of several species of *Aeromonas* from patients with gastroenteritis, and these have been extensively reviewed (Altwegg & Geiss, 1989; Janda, 1991; Joseph, 1996). There has been considerable debate as to whether the mesophilic aeromonads are primary enteropathogens, prompted largely by failure to establish significant infection in volunteer studies. In a study in which 57 people were challenged using five strains of *A. hydrophila* with doses ranging from 10^4 to 10^{10} organisms, only two individuals developed diarrhoea—one had mild diarrhoea after a dose of 10^9 organisms and the other developed moderate diarrhoea after a dose of 10^7 (Morgan et al., 1985). The value of these data is limited, as the strains used were poorly characterized and some were not demonstrably enterotoxigenic (Gosling, 1996). However, there have been reports of laboratory-acquired infections in microbiologists who (unintentionally) ingested significant doses of *Aeromonas* and developed self-limiting diarrhoea (Joseph, 1996).

Understanding the clinical significance of enteric isolates of *Aeromonas* has been further complicated by the fact that some studies have demonstrated similar isolation frequencies from symptomatic and asymptomatic adults (Altwegg & Geiss, 1989), while others have shown significant correlations between diarrhoea

Table 2. Relative frequency of occurrence of human infections associated with mesophilic *Aeromonas*^a

Type of infection	Characteristics	Relative frequency ^b
<i>Diarrhoea</i>		
Secretory	Acute watery diarrhoea, vomiting	Very common
Dysenteric	Acute diarrhoea with blood and mucus	Common
Chronic	Diarrhoea lasting more than 10 days	Common
Choleraic	"Rice water" stools	Rare
<i>Systemic</i>		
Cellulitis	Inflammation of connective tissue	Common
Myonecrosis	Haemorrhage, necrosis with/without gas gangrene	Rare
Erythema gangrenosum	Skin lesions with necrotic centre, sepsis	Uncommon
Septicaemia	Fever, chills, hypotension, high mortality	Fairly common
Peritonitis	Inflammation of peritoneum	Uncommon
Pneumonia	Pneumonia with septicaemia, sometimes necrosis	Rare
Osteomyelitis	Bone infection following soft-tissue infection	Rare
Cholecystitis	Acute infection of gallbladder	Rare
Eye infections	Conjunctivitis, corneal ulcer, endophthalmitis	Rare

^a Modified from Janda & Duffey, 1988, and Nichols et al., 1996

^b Frequency of occurrence relative to all cases of *Aeromonas* infection

and enterotoxin-producing *Aeromonas* spp. (Gracey, Burke & Robinson, 1982; Bloom & Bottone, 1990; Joseph, 1996). Seasonal variations in isolation of *Aeromonas* from stools has also been reported, with highest recovery during the warmer months (Burke et al., 1984a; Moyer, 1987). The available evidence indicates that people are generally unaffected by enteric *Aeromonas* and that aeromonads may be a natural part of the gut flora, either transiently or in the longer term. A number of factors, including age, immunocompetence, infection dose, underlying illness, and expression of sufficient virulence factors by the infecting organism, affect the ability of *Aeromonas* spp. to cause disease (Nichols et al., 1996).

Although the pathogenesis of *Aeromonas* infections remains poorly understood, mesophilic *Aeromonas* spp. can express a range of virulence factors (Gosling, 1996), including attachment mechanisms and production of a number of toxins. Several studies have demonstrated that strains of *A. hydrophila* produce lectins and adhesins which enable adherence to epithelial surfaces and gut mucosa (Gosling, 1996). Additionally, two types of pili have been characterized from Hep-2-adherent *A. hydrophila* (Carrello et al., 1988; Gosling, 1996), and invasion of Hep-2 cells by faecally derived *A. hydrophila* has also been reported (Lawson, Burke & Chang, 1985).

Species of *Aeromonas* are capable of expressing a number of extracellular toxins and enzymes (Gosling, 1996; Howard, MacIntyre & Buckley, 1996). Early characterization of the toxins, however, resulted in confusion regarding their

number and activities. The primary toxins produced are haemolysins, of which the most significant is aerolysin, expressed by many strains of *A. hydrophila* and *A. sobria* (Janda, 1991; Howard, MacIntyre & Buckley, 1996). This is a heat-labile β -haemolysin, which exhibits phospholipase A and C activity. It is a pore-forming cytotoxin able to insert into the cell membrane bilayer causing leakage of cytoplasmic contents. Haemolytic enterotoxins have been reported by some authors (Chopra, Houston & Kurosky, 1991; Gosling, 1996). A weak haemolysin, glycerophospholipid:cholesterol acyltransferase (GCAT), has been characterized from *A. hydrophila* and *A. salmonicida* (Howard, MacIntyre & Buckley, 1996); other haemolysins may also exist, but need to be isolated and purified before haemolytic activity can be confirmed. In addition, at least one cytotoxic enterotoxin with similar activity to cholera toxin has been demonstrated (Ljungh, Eneroth & Wadström, 1982; Gosling et al., 1992; Gosling, 1996), and there may be several. Evidence for plasmid-encoded expression by *A. hydrophila* and *A. caviae* of a cytotoxin similar to Shiga-like toxin 1 has been reported (Haque et al., 1996). Species of *Aeromonas* also produce a range of cell-surface and secreted proteases which probably enhance virulence (Gosling, 1996). Expression of virulence factors, including haemolysins and proteases, by aeromonads has been shown to be influenced by environmental temperature (Eley, Geary & Wilcox, 1993; Mateos et al., 1993).

There is abundant evidence to suggest associations between mesophilic aeromonads and diarrhoea, and production of enterotoxins has been demonstrated. Further work is needed to clarify the pathogenic mechanisms of *Aeromonas* spp. and substantiate the causative role of these organisms in gastroenteritis. There is also a need for reliable data on human infective doses for well-defined strains of putative enteropathogenic aeromonads, pending the establishment of an appropriate animal model for the study of *Aeromonas*-associated diarrhoea.

Health significance of *Aeromonas* in drinking-water

The health significance of detecting mesophilic aeromonads in public water supplies is not well understood: no clearly defined point-source outbreak has been documented and establishing epidemiological links is difficult.

Reports from Australia (Burke et al., 1984a; 1984b) have suggested that there may be a connection between cases of *Aeromonas*-associated diarrhoea and the numbers of *Aeromonas* in the drinking-water. In later studies following increases in numbers of aeromonads in treated water in the Netherlands, some of the strains isolated demonstrated strong cytotoxic properties (van der Kooij, 1988). Following a review of data available at the time, the health authorities in the Netherlands in 1985 introduced "indicative maximum values" for *Aeromonas* densities in drinking-water. The values were based on a national survey of aeromonads in drinking-water in the Netherlands and have been defined as follows: 20 cfu/100 ml as a median value over a 1-year period in water leaving

the treatment facility; 200 cfu/100 ml as the 90th-percentile value of the *Aeromonas* counts of drinking-water collected from the distribution system in a 1-year period (Trouwborst, 1992). It should be noted that these values were based on assessment of achievability in the Netherlands, motivated by a precautionary approach, and not on the public health significance of the occurrence of *Aeromonas* in drinking-water.

Virulence factors

Several studies have demonstrated that many mesophilic aeromonads isolated from drinking-water can exhibit toxigenic factors. Millership, Barer & Tabaqchali (1986) found that cytotoxicity was demonstrated by 28% of *Aeromonas* isolates (mainly *A. hydrophila*) from chlorinated and unchlorinated drinking-water but by none of the strains of *A. caviae* (which represented 50% of the isolates). More recently Holmes, Niccolls & Sartory (1996) found that 20% of *Aeromonas* isolates exhibited phenotypic characteristics associated with enterotoxicity; of these, 75% were *A. hydrophila*, 14% *A. sobria*, 9% *A. caviae*, and the remainder *A. schubertii*. In contrast, Burke et al. (1984b) reported that 61% of aeromonads isolated from an unchlorinated municipal water supply in Australia were enterotoxigenic, and 64% produced haemolysins. Notermans et al. (1986) found that all of 26 drinking-water isolates of *A. hydrophila* and 9 of 22 isolates of *A. sobria* exhibited the haemolytic enterotoxin Asao toxin and cytotoxicity to Vero cells, while none of 14 isolates of *A. caviae* was positive. Similarly, Krovacek et al. (1992) found that 100% of *A. hydrophila* and 70% of *A. sobria* in Swedish chlorinated and unchlorinated drinking-water were haemolytic, but that less than 30% of the isolates were enterotoxigenic. Kirov et al. (1994) found that 53.6% of isolates of *A. hydrophila* hybridization group 1 (HG1) and 55.9% of HG3 from water expressed two or more virulence factors.

Epidemiology

Despite the association of virulence factors with drinking-water aeromonads, there is increasing evidence that strains isolated from the environment generally belong to different groups from strains associated with gastroenteritis. Havelaar et al. (1992) typed 187 *Aeromonas* isolates from human diarrhoeal stools and 263 from drinking-water. There was little similarity between the strains from stools and those from drinking-water. This was particularly true of *A. caviae*, which was the dominant aeromonad in both sets of samples. Other studies have indicated that *A. hydrophila* prevalence may be related to hybridization groups. Both Kirov et al. (1994) and Hänninen (1994) found that HG1 was associated with clinical specimens, while HG3—and to a lesser extent HG2—predominated in water and environmental samples. It appears that this may be reflected in the maximum growth temperatures (t_{max}) of the homology groups. Hänninen, Salmi & Siitonen (1995) have reported that hybridization groups of *Aeromonas* associated with

clinical samples (HG 1, 4, 9/10 and 13) generally had a t_{max} of 40–44 °C, while isolates from freshwater (HG 3 and 11) had t_{max} values between 36.5 and 37.5 °C.

It has been claimed that drinking-water supplies are responsible for the increased incidence of *Aeromonas*-associated gastroenteritis. Ghanem, Mussa & Eraki (1993) considered that, since 90% of the domestic water supplies in Cairo were positive for aeromonads, and that 56% of isolates produced enterotoxins, the supplies were a major source of *Aeromonas* infections. Investigating a case of long-term diarrhoea in a child aged 18 months, Krovacek et al. (1989) concluded that the cause was *A. hydrophila* from a private, unchlorinated well in which counts ranged from 70 cfu/100 ml to 6.4×10^4 cfu/100 ml. The majority of isolates were enterotoxin-producers.

Although these reports (Burke et al., 1984a, 1984b; Krovacek et al., 1989; Ghanem, Mussa & Eraki, 1993) indicate a possible relationship between *Aeromonas* in drinking-water and increased incidence of aeromonad-related illness, the evidence is tenuous. In one case comparing the typing of faecal and water isolates, the two groups proved to be unrelated (Moyer et al., 1992). Following a number of cases of diarrhoea in children using a small community water supply, *Aeromonas* was isolated from water-treatment and distribution samples; ribotyping and DNA hybridization showed that isolates from faeces were of different ribotypes and DNA hybridization groups (HG 1 and 4) from drinking-water isolates (predominantly HG 2, 3 and 5A) (Moyer et al., 1992).

Monitoring and assessment

Routine monitoring for *Aeromonas* in piped and non-piped water supplies cannot be justified on the basis of present knowledge of their role in human infection. Monitoring or periodic surveys may be required in some circumstances, for instance where especially vulnerable populations are exposed; and further research is justified.

Membrane filtration is the procedure most commonly used for the enumeration of *Aeromonas* from treated water; it employs a variety of culture media, most of which contain ampicillin. For drinking-water, the most widely used medium is ampicillin–dextrin agar (ADA) (Havelaar, During & Versteegh, 1987; Havelaar & Vonk, 1988). An alternative, which gives the same selectivity and sensitivity, is Ryan's *Aeromonas* medium (Holmes & Sartory, 1993). These media, however, contain selective agents and are nutrient-rich, and their use may result in low recovery of some aeromonads from low-nutrient or chlorinated waters, weighting any data in favour of the more robust, rapidly growing strains (Gavriel & Lamb, 1995; Holmes, Niccolls & Sartory, 1996). The incubation regime is typically 28–30 °C for 24–48 hours. *Aeromonas* species are sensitive to the presence of copper at concentrations as low as 10 µg/l, and a complexing agent (50 mg/l sodium ethylenediamine tetraacetate, Na₂EDTA, or sodium nitrilotriacetate, Na₃N₃TA) should therefore be added to samples from domestic and other properties containing copper piping to reduce die-off (Versteegh et al., 1989;

Schets & Medema, 1993). Pre-enrichment with alkaline peptone water before subculturing to selective media has proved successful for recovery of *Aeromonas* from water (e.g. well water) in which the number of organisms is low (Moyer et al., 1992).

Several different media have been used for the recovery of *Aeromonas* from environmental waters; m-aeromonas agar (Rippey & Cabelli, 1979), ADA, starch–ampicillin agar (Palumbo et al., 1985), pril–xylose–ampicillin agar (Rogol et al., 1979) and SGAP-10C agar (Huguet & Ribas, 1991) are the most widely used (Moyer, 1996). All these media contain ampicillin and some have been shown to result in under-recovery of certain species such as *A. sobria*, *A. veronii* and *A. schubertii* (Gavriel & Lamb, 1995).

Primary identification of isolates as members of the genus *Aeromonas* is relatively simple. Many laboratories should be able to assign the mesophiles to one of the classical complexes (*A. hydrophila*, *A. caviae*, and *A. sobria*), but identification to phenospecies or genospecies level through biochemical testing can be problematic because of taxonomic complexities within the genus (Millership, 1996).

As yet, there are few published immunological or molecular methods for detecting mesophilic *Aeromonas* spp. in water compared with the number currently available for *Escherichia coli* and other members of the Enterobacteriaceae. A polymerase chain reaction (PCR) procedure based on 16S rRNA (Khan & Cerniglia, 1997) has been successfully used for the detection of *A. caviae* and *A. trota* in seafood and water samples, and PCR amplification of 16S rDNA sequences has been used to identify environmental isolates of *Aeromonas* (Dorsch et al., 1994).

Control

Environmental occurrence

Aeromonads are ubiquitous in aquatic environments and readily isolated from both nutrient-rich and nutrient-poor environments (Holmes, Niccolls & Sartory, 1996). Typical numbers of *Aeromonas* in a range of aquatic environments are given in Table 3. As *Aeromonas* are autochthonous to fresh and marine waters their recovery is to be expected. However, increasing levels of pollution may result in substantially greater populations, and may also affect distribution of the organisms (Holmes, Niccolls & Sartory, 1996). Several studies have shown that *A. caviae* tends to predominate in waters with a high degree of organic loading (Araujo, Arribas & Pares, 1991; Stecchini & Domenis, 1994); *A. caviae* and *A. hydrophila* are almost equally distributed in less polluted waters, and *A. sobria* becomes more frequent in unpolluted and brackish waters (Holmes, Niccolls & Sartory, 1996). Aeromonad densities have also been related to trophic status, and populations in some waters have a seasonal variation, with highest numbers occurring in the warmer months (Rippey & Cabelli, 1989). Relationships between aeromonad densities and parameters relating to trophic status or sewage

Table 3. Typical numbers of *Aeromonas* species in aquatic environments^a

Environment	Typical counts (cfu/ml)
Domestic sewage sludge	>10 ⁸
Crude sewage	10 ⁶ –10 ⁸
Treated sewage	10 ³ –10 ⁵
Wastewater	10 ² –10 ⁷
Rivers receiving sewage discharges	10–10 ⁴
Clean rivers, lakes, storage reservoirs	1–10 ²
Seawater	10 ⁻² –10 ²
Drinking-water, post-treatment	10 ⁻² –10
Drinking-water, in distribution system	10 ⁻² –10 ³
Groundwaters	<1

^a Reproduced, with permission, from Holmes, Niccolls & Sartory (1996)

contamination will vary according to the site, season and region (Rhodes & Kator, 1994).

Effects of drinking-water treatment

As aeromonads can occur in large numbers in some water sources (particularly lowland rivers and reservoirs), there is potential for them to enter distribution systems if water treatment is ineffective (Holmes, Niccolls & Sartory, 1996). A survey of a treatment works in Belgium demonstrated the following cumulative reduction of aeromonads at different stages of the treatment process under summer and winter conditions (Meheus & Peeters, 1989):

following flocculation/sedimentation	30–60%
following rapid sand filtration	70–90%
following granular activated carbon	80–90%
following hyperchlorination/direct filtration	99–100%

In the same study reductions following slow sand filtration were 98–100%. Neither the mode of cleaning nor the age of the filters appeared to influence the elimination of *Aeromonas*.

Studies of five water-treatment plants in Belgium (Huys et al., 1995; Kersters et al., 1995) reported a mean reduction of 99.7% in aeromonad numbers following flocculation–decantation and chlorination. Slow sand filtration reduced aeromonad numbers by 98.9%. Increased levels of *Aeromonas* were obtained from the effluents of activated carbon filters. Following slow sand filtration at a plant that treated surface water, there was a marked shift from a predominance of *A. hydrophila* and *A. sobria* to a predominance of *A. caviae*.

In a study on the impact of the type of material used for rapid gravity filters at a treatment works (Holmes, Niccolls & Sartory, 1996), coagulation and

clarification resulted in a mean reduction of 90% and aeromonads were undetectable after post-clarifier chlorination. There was a marked difference between sand-based rapid gravity filters and those employing granulated activated carbon (GAC). *Aeromonas* were recovered on only one occasion from the sand filters as chlorine levels were maintained through the beds. However, chlorine was rapidly removed by the activated carbon filters resulting in concentrations of less than 0.1 mg/l in the effluent. *Aeromonas* were recovered throughout the year, with greater numbers between July and September when water temperatures were highest.

Low numbers of *Aeromonas* have been reported in the final waters of 20 plants treating surface waters and groundwaters in the Netherlands (Havelaar, Versteegh & During, 1990). The maximum count of 470 cfu/100 ml was recorded from a plant treating deep aerobic groundwater. The high counts obtained at some works were often associated with filter beds with long operational periods (over 25 years) without replacement of filter material, or with filter units that were operated intermittently to meet variable water demand.

Clearly, water treatment can significantly reduce levels of *Aeromonas*, but these bacteria are capable of establishing significant populations in GAC-based treatment processes. Low numbers may be recovered from the final waters of water-treatment plants that meet water quality standards for the hygienic indicator organisms.

Changes in piped distribution systems

Aeromonads are readily isolated from municipal drinking-water systems, sometimes at quite high levels (Havelaar, Versteegh & During, 1990; Krovacek et al., 1992; Stelzer et al., 1992; Holmes, Niccolls & Sartory, 1996). Knöchel & Jeppesen (1990) examined drinking-water in Denmark and found that only 28% of samples were positive, with counts ranging from 1 to 40 cfu/100 ml; *A. hydrophila* made up 97% of isolates. In contrast, Ghanem, Mussa & Eraki (1993) reported that 90% of domestic water supplies in areas of Cairo contained *Aeromonas*, while from a survey of three distribution systems in Sweden, Krovacek et al. (1992) reported that 85% of samples were positive for presumptive *Aeromonas*, with a maximum count of 860 cfu/100 ml. *A. hydrophila* accounted for 67% of the strains isolated; the remainder were *A. sobria*. Stelzer et al. (1992) recorded a maximum count of 240 *Aeromonas*/100 ml in a drinking-water supply in Germany, with an isolation frequency for *A. hydrophila* of 37% and for *A. sobria* of 57%. The highest counts were obtained from points furthest (>10 km) from the treatment works. Havelaar, Versteegh & During (1990) reported regrowth of aeromonads in 16 of 20 distribution systems examined in the Netherlands. Geometric mean counts varied between 1 and 440 cfu/100 ml; a maximum count of 3300 cfu/100 ml was obtained from a system supplying water abstracted from a river source. Growth of *Aeromonas* generally occurred in the peripheral parts of distribution systems and was associated particularly with drinking-water

derived from anaerobic groundwaters containing methane. *Aeromonas* densities usually showed a seasonal pattern, with peak values occurring in late summer when water temperatures were highest. All three classical species were recovered during the survey, with either *A. hydrophila* or *A. caviae* tending to predominate. Although *A. sobria* predominated in one system, this species tended to be recovered in low numbers only. LeChevallier et al. (1982) reported that the aeromonads that occurred in 27% of samples taken over an 18-month period from a chlorinated supply in Oregon, USA, consisted solely of *A. sobria*.

Although species of mesophilic *Aeromonas* are commonly resident in drinking-water distribution systems, there are few data on the factors affecting their occurrence. However, it is generally reported that higher rates of isolation and larger populations occur during the warmer months and at the peripheries of distribution systems (LeChevallier et al., 1982; Havelaar, Versteegh & Doring, 1990; Stelzer et al., 1992; Holmes, Niccolls & Sartory, 1996). For chlorinated water, Burke et al. (1984a) reported that *Aeromonas* occurrence was positively correlated with water temperature and negatively correlated with residual chlorine levels. A seasonal variation in mean *Aeromonas* counts closely paralleled mean water temperature in samples that were either unchlorinated or had free chlorine values consistently below 0.3 mg/l. Isolation of *Aeromonas* spp. from drinking-water lacking chlorine was generally associated with water temperatures greater than 14.5°C.

A study of a large supply system in central England resulted in a model of best fit relating the probability of occurrence of *Aeromonas* to temperature, free chlorine, and age of water (Holmes, Niccolls & Sartory, 1996). The probability of occurrence of *Aeromonas* increased significantly when the mean seasonal temperature exceeded 14°C and this was exacerbated where the mean free chlorine concentration fell below 0.1 mg/l. The impact of water age was significant only when the mean free chlorine level was less than 0.1 mg/l. There was no relationship between *Aeromonas* incidence and coliforms or heterotrophic plate counts. Strains of *A. hydrophila*, *A. caviae*, and *A. sobria* isolated from drinking-water can grow at 4°C (Holmes, Niccolls & Sartory, 1996) and are thus capable of growth throughout the year in many geographical areas.

It is apparent that water temperature and free chlorine are factors that significantly influence the growth of *Aeromonas* in drinking-water supplies. Mesophilic aeromonads are nutritionally versatile. Studies using a drinking-water isolate of *A. hydrophila* demonstrated its ability to utilize a variety of organic compounds, including carbohydrates, amino acids, carboxylic acids, and long-chain fatty acids, at low concentrations (10 µg/l) (van der Kooij & Hijnen, 1988; van der Kooij, 1991); mixtures of compounds at individual concentrations of 0.1 or 1 µg/l (expressed as carbon) enhanced growth. These results demonstrate that aeromonads are capable of growth in the presence of the low concentrations of nutrients that would be available from biofilms and sediments within distribution systems. Thus the organic carbon content (assimilable organic carbon, AOC,

or biodegradable organic carbon, BDOC) of the water would also be expected to be a determining factor in the occurrence of these organisms, but this has yet to be studied within water distribution systems. In a study on biofilm formation characteristics, various types of drinking-water demonstrated large differences in biofilm formation rates (van der Kooij & Veenendaal, 1993). A highly significant correlation between biofilm formation rate of groundwater-derived drinking-water and *Aeromonas* density in drinking-water during distribution has also been demonstrated (van der Kooij et al., 1995).

Typically, *Aeromonas* in drinking-water in distribution systems has been controlled by increased disinfection, and it appears that free cells of *Aeromonas* are relatively susceptible to the common chlorine-based disinfectants. Knöchel (1991) found that strains of *A. hydrophila*, *A. sobria*, *A. caviae*, and *A. veronii* were generally more susceptible to chlorine and monochloramine than coliforms and pseudomonads, and Medema et al. (1991) found that laboratory-grown and environmental *Aeromonas* were also susceptible to chlorine dioxide. Despite this relative susceptibility to chlorine-based disinfectants, controlling the numbers of aeromonads in a distribution system may require some considerable time and chlorine concentrations in excess of 0.2 mg/l (Edge & Finch, 1987). This is probably due to association of the organisms with biofilms. Mackerness, Colbourne & Keevil (1991) found that *A. hydrophila* became readily established within a mixed heterotrophic bacterial biofilm and was unaffected by addition of 0.3 mg/l monochloramine. There was evidence that the biofilm-associated *A. hydrophila* would also survive 0.6 mg/l monochloramine, which was sufficient to eradicate biofilm-associated *E. coli*. These data indicate that, although free cells of *Aeromonas* may be relatively susceptible to disinfection, populations associated with biofilms may survive high chlorine dosing. A key mechanism for the control of aeromonads in drinking-water is therefore the removal of biodegradable compounds (i.e. improving the biostability of the water). Where reasonably practical, limiting the concentrations of biodegradable compounds (e.g. treatment with granular activated carbon treatment or, for anaerobic groundwaters, by aeration) may be the preferred option. Such measures would also help to control the regrowth of heterotrophic bacteria and the proliferation of invertebrates within the distribution system.

Conclusions and recommendations

Health risk assessment

Although aeromonads are frequently isolated from drinking-water systems, and some strains may exhibit enterotoxigenic properties, further epidemiological studies are required to ascertain any significance in relationships between cases of *Aeromonas*-associated diarrhoea and presence of these organisms in drinking-water. Current evidence indicates that the predominant aeromonads typically

found in drinking-water do not belong to the same DNA homology groups as those isolated from cases of gastroenteritis. It also appears that, if species of *Aeromonas* are primary enteropathogens, high numbers are required to initiate disease. As numbers in drinking-water are generally low compared with those found in foods (10^3 – 10^5 cfu/g), treated drinking-water probably represents a very low risk. The virulence of enterotoxigenic *Aeromonas* for risk groups (newborn infants and immunocompromised individuals), however, remains to be ascertained. To date there is no firm evidence that direct transmission occurs via drinking-water, but in the absence of more definitive proof of their public health significance it would be advisable to control excessive numbers of aeromonads in drinking-water supplies.

Risk management strategies

The mesophilic aeromonads are a ubiquitous component of the natural bacterial flora of aquatic environments. When temperature and nutrient conditions allow, they can rapidly proliferate in unchlorinated drinking-water supply systems and where chlorine residuals tend to be low (e.g. in the extreme parts of extensive distribution systems). The key factors in controlling *Aeromonas* proliferation are temperatures below 14°C (although the organisms are capable of growth at 4°C), free chlorine residuals above 0.1–0.2 mg/l, and the limitation of organic carbon compounds that would serve as nutrients. Control of the development of biofilms within water supply systems will reduce, but not prevent, the proliferation of *Aeromonas*. As *Aeromonas* are associated with biofilm development, significant increases in numbers in a drinking-water supply are indicative of a general deterioration of bacteriological quality. The increasing use of granulated activated carbon in water treatment may allow proliferation and dissemination of *Aeromonas*. Limiting the numbers of aeromonads released into distribution systems thus requires effective management of filter beds and maintenance of adequate final chlorination. Control of aeromonad numbers in piped distribution systems is achieved primarily by limiting regrowth possibilities; this will also limit the numbers of heterotrophic bacteria and improve the efficacy of chemical disinfection in the distribution system.

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Enteric hepatitis viruses¹

Description

The term “hepatitis viruses” refers to a diverse group of viruses all of which have the human liver as the primary target of replication and give rise to hepatitis or inflammation of the liver. Their replication may result in mass destruction of liver cells. Consequences include failure of the liver to fulfil basic functions such as removal of bilirubin from the circulatory blood system. Bilirubin is a red pigment released from red blood cells as they break down and are replaced by new cells. Excessive accumulation of the pigment in the bloodstream is manifest as yellow coloration of sites such as the eyes and the palms of the hands. This condition is known as jaundice and is also marked by dark urine and stools resulting from the excretion of bilirubin. Another typical consequence of massive liver cell damage is release into the bloodstream of liver enzymes, including alanine transaminase (ALT) and aspartate transaminase (AST). Serum levels of these enzymes are used to diagnose hepatitis (Zuckerman & Thomas, 1993).

Hepatitis may also be caused by other systemic pathogens such as cytomegalovirus, yellow fever virus, and *Leptospira* bacteria, although the liver is not the primary or only target of these organisms. Liver cell damage and jaundice may also be caused by toxic compounds, including alcohol.

Since the clinical symptoms caused by hepatitis viruses are very similar, and some of the viruses only emerged on a large scale in recent years, distinction of different aetiological agents has been progressively accomplished only since the 1960s. The first two hepatitis viruses that were distinguished were simply

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designated A and B, because at that time there was no indication of more. As new hepatitis viruses were discovered, the alphabetical nomenclature was retained. The range has already reached G, and there are indications of more hepatitis viruses. Unfortunately, this non-descriptive system of alphabetical nomenclature is confusing to the non-expert in the field. The nomenclature is abbreviated as HAV to HGV for hepatitis A to G viruses.

Hepatitis viruses are divided into two basic groups, some distinctive features of which are summarized in Table 4.

The group referred to as enteric hepatitis viruses consists of HAV, HEV, and HFV. The parenterally transmitted or bloodborne hepatitis viruses form the second group and consist of HBV, HCV, HDV, and HGV. Enteric hepatitis viruses are transmitted primarily by the faecal–oral route: explosive epidemics of HAV, and particularly of HEV, usually result from faecal contamination of water or food. The parenterally transmitted viruses are transmitted primarily by blood and blood products—by medical transfusion, as well as by sexual intercourse, use of contaminated medical instruments such as syringes and needles, and even by tattooing and insect bites. There is no evidence that parenterally transmitted viruses are of significant concern to water quality. HBV seems to be inactivated

Table 4. Classification of hepatitis viruses

Hepatitis virus	Characteristic features ^a
<i>Enteric</i>	
Hepatitis A (HAV)	Family Picornaviridae, non-enveloped 27–32 nm icosahedral particles; ssRNA, no chronic infection or carrier state, vaccines available, worldwide, low mortality
Hepatitis E (HEV)	Family Calciviridae, non-enveloped 25–35 nm icosahedral particles, ssRNA, no chronic infection or carrier state, no vaccine available; restricted geography, high mortality in pregnant women
Hepatitis F (HFV)	Unclassified, non-enveloped 27–37 nm icosahedral particles, dsDNA, no chronic infection or carrier state, no vaccine available, apparently restricted geography and low mortality
<i>Parenterally transmitted</i>	
Hepatitis B (HBV)	Family Hepadnaviridae, enveloped 42 nm spherical particles; dsDNA, sometimes chronic carrier state, vaccines available, worldwide, sometimes leads to fatal liver cancer
Hepatitis C (HCV)	Family Flaviviridae; enveloped 50 nm spherical particles, ssRNA, chronic infection in >50% of cases; no vaccine available, worldwide
Hepatitis D (HDV)	Classified as sub-viral agent, enveloped 35–37 nm spherical particles; ssRNA, often chronic infection; no specific vaccines, restricted geography, defective virus dependent on HBV
Hepatitis G (HGV)	Family Flaviviridae, enveloped 35–37 nm spherical particles, ssRNA, often chronic infection, no vaccine available, worldwide

^a ss = single-strand, ds = double-strand

by enzymes produced by bacteria in the gastrointestinal tract and water resources (Grabow et al., 1975), and infectious HBV is therefore rarely, if ever, detectable in faeces, water, or food. This seems also to be true of HCV, HDV, and HGV, and so there is no further discussion of these viruses here.

The global public health impact of enteric hepatitis virus infections is immense. No attempt has yet been made to calculate meaningful figures because of the effect of variables such as the relationship between infections and standard of living, subclinical cases, and under-reporting. However, enough data exist to give an indication of general trends. For instance, statistics for the United States indicate that viral hepatitis was the second most frequently reported infection in 1990 after sexually transmitted diseases (Zuckerman & Thomas, 1993). In 1981, 57 929 cases of viral hepatitis (25.3/100 000 population) were reported, of which 45% were hepatitis A. Records show 31 441 cases of hepatitis A (12.64/100 000 population) in 1990 and 31 582 cases in 1995 (American Academy of Pediatrics Committee on Infectious Diseases, 1996). The direct and indirect cost per clinical case of viral hepatitis A infection has been estimated at US\$ 1000 for individuals aged up to 18 years and US\$ 2100 for those older than 18 years. A study of a foodborne outbreak of hepatitis A in Denver, Colorado, in 1992 with 43 secondary cases and potential exposure of approximately 5000 people, revealed direct medical costs associated with infected patients of US\$ 46 064. This was only 7% of the US\$ 689 314 spent on controlling the disease (epidemiological studies to locate the source of the outbreak, interventions to prevent infection of potentially exposed individuals, and related expenses) (Dalton et al., 1996). Extrapolation of these cost estimates to episodes such as the 1988 shellfish-associated outbreak in Shanghai, China, with close to 300 000 clinical cases of hepatitis A and 32 deaths (attack rate 4083/100 000 population), yields dramatic figures. The United States cost figures for hepatitis A are likely to be representative of developed countries. Hepatitis A morbidity patterns are different for developing countries, however, where subclinical infection in childhood years may be almost universal (Zuckerman & Thomas, 1993). Although no corresponding cost estimates have yet been reported for viral hepatitis E, the costs may be even higher than for hepatitis A, since the infection is generally contracted later in life, with a relatively high incidence of clinical cases and mortality in pregnant women. The financial burden of hepatitis E in countries where it is the most common cause of viral hepatitis (Grabow, 1997) is therefore likely to be extremely high. Likewise, the public health burden of single outbreaks, such as those with more than 100 000 cases in China between 1986 and 1988, and an estimated 79 000 cases in India in 1991 (Grabow, 1997), is enormous.

Hepatitis A virus

Of the three enteric hepatitis viruses currently recognized, HAV has the longest and best known history. Initially HAV was known as the “infectious” or “epidemic” hepatitis virus, because of its typical association with epidemics caused

by contaminated water and food worldwide (Mosley, 1959; Grabow, 1976; Zuckerman, 1983). Extreme examples include the 1988 outbreak of some 300 000 cases in Shanghai, China, caused by the consumption of clams harvested from a bay polluted with sewage from a community that had experienced an epidemic of hepatitis A (Halliday et al., 1991).

HAV is a typical member of the family Picornaviridae. It has a non-enveloped icosahedral capsid of diameter 25–35 nm and a single-strand RNA genome. HAV shares many features with members of the genus *Enterovirus*, such as polioviruses and coxsackieviruses, and has at some time been classified as enterovirus type 72. More recently, however, HAV has been classified in its own genus, known first as *Heparnavirus* and then as *Hepatovirus*.

Initial multiplication of picornaviruses like polioviruses, coxsackieviruses, and HAV takes place in the lymphoid tissue of the pharynx, and these viruses are detectable in throat swabs and sputum specimens during early stages of infection. The predominant site of replication is the lymphoid tissue of the gut, and the viruses are therefore typically detectable in stool specimens. Only in a small percentage of cases do enteroviruses proceed to infect the central nervous system, and HAV to infect the liver. HAV replication in the liver causes damage to liver cells, which is known as hepatitis. In immune individuals, however, circulating antibodies prevent HAV from infecting the liver.

Like many picornaviruses, HAV tends to cause infections that are mild or without clinical symptoms in children. The incidence of infection is closely linked with hygiene and sanitation conditions, and most people in developing countries contract infections during early childhood. Typical clinical symptoms of infection are predominantly seen in adults. Although mortality is generally less than 1%, the disease may be quite severe and incapacitating; there may be substantial liver damage, and regeneration of the liver cells takes time (Zuckerman & Thomas, 1993). Patients may feel ill and be confined to bed for up to 6 weeks or more; they usually lack interest in foods that depend heavily on liver functions for digestion. The severity of illness and mortality may be dependent on underlying conditions such as immunodeficiencies and malnutrition, and on the general state of health.

There seems to be only one antigenic type of HAV, which elicits lifelong immunity. In adult populations of developing countries and communities, immunity to HAV may exceed 95%, in contrast to developed countries and communities where levels of immunity may be less than 50% (Iwarson, 1992; Sathar et al., 1994; Tucker et al., 1996). People from developed countries who visit developing areas are therefore exposed to a high risk of infection. HAV typically occurs in all parts of the world and, beyond the link to standards of hygiene and sanitation, gives no indication of geographical preferences.

Unlike closely related viruses such as polioviruses and some coxsackieviruses, HAV is not readily detectable by routine cell culture procedures. Many questions about the epidemiology of the virus, as well as about its occurrence and behaviour in the environment, therefore remain unanswered. However, there is

little doubt that the virus is highly infectious and can cause explosive outbreaks when present in water or food. In addition, it is relatively resistant to unfavourable environmental conditions, including water treatment and disinfection processes. High infectivity has been demonstrated for closely related viruses such as echoviruses and poliovirus vaccine strains in experiments using human volunteers (Grabow, 1996). In terms of its transmission by water and food, HAV is even more infectious, as shown by the high risk of infection associated with faecally polluted water and food—even supplies that meet generally accepted quality limits for coliform indicator bacteria (Bosch et al., 1991a, 1991b). Closely related viruses such as polioviruses, coxsackieviruses, and echoviruses are less frequently associated with waterborne transmission (Grabow, 1996).

Much of the available information on HAV derives from experiments with human volunteers. Well-known studies include those of Neefe et al. (1947) carried out during the Second World War in attempts to control a disease that is notorious for its devastating impact on troops and civilians alike during times of war. This work was followed some years later by experiments on another group of human volunteers, which resulted, among other things, in the first distinction between HAV and HBV (Krugman & Giles, 1970).

An important step forward was the visualization of the virus by immune electron microscopy, and then propagation of the virus in certain primates, notably marmosets and chimpanzees (see Grabow et al., 1981). This led to the development of immunological assays for detection of the virus and its antibodies (Coulepis et al., 1985). Another milestone was the discovery that HAV can replicate slowly in certain cell cultures (Frösner et al., 1979), and this led to the establishment of cell-culture-adapted strains (Gust et al., 1985). Although these adapted strains may differ in some respects from wild-type HAV, they made it possible to study the behaviour of the virus in the environment and to develop vaccines, which are now freely available. The advent of molecular techniques, notably gene probe hybridization and the polymerase chain reaction (PCR), led to the development of sensitive techniques for detection of the virus (Dubrou et al., 1991; Deng, Day & Cliver, 1994; Tsai et al., 1994).

The incubation period of hepatitis A may vary from 15 to 45 days, with a mean of 30 days (Reid & Dienstag, 1997), i.e. some 10 days less than the incubation period of hepatitis E. Faecal excretion of HAV begins late in the incubation period, peaks just before onset of clinical symptoms of disease (usually the appearance of dark urine), and falls to barely detectable levels as the clinical illness evolves. The virus is present in blood in relatively low numbers for about 7–14 days, with a peak before the onset of clinical symptoms (Zuckerman & Thomas, 1993).

Typically, faecal–oral transmission of HAV is the result of personal contact or the consumption of faecally polluted water or food. A common sequence of events is for one member of a household to contract the infection from contaminated water or food, or from contact with an infected individual outside the home, and then to infect other members of the household by personal contact.

Household infection rates of up to 95% have been recorded (Villarejos et al., 1982). This secondary transmission is difficult to predict or prevent because the virus is transmitted within the family before infection of the primary case is evident (Halliday et al., 1991; Zuckerman & Thomas, 1993). Although a variety of non-human primates are susceptible to HAV under experimental laboratory conditions, and transmission of the virus from chimpanzees to humans during close contact is well documented (Grabow et al., 1981; Zuckerman & Thomas, 1993), there is no evidence that animals may serve as a significant reservoir for HAV.

Hepatitis E virus

The existence of HEV was confirmed in the late 1970s and early 1980s (Wong et al., 1980), after it became evident that there was a hepatitis virus other than HAV and HBV. Infection with the virus was initially referred to as enterically transmitted (or epidemic) non-A, non-B hepatitis. It eventually transpired that HEV had for many years been mistaken for HAV, because the two viruses share certain basic clinical and epidemiological properties (Grabow et al., 1994; Purcell, 1996). Both are transmitted primarily by the faecal–oral route, and are often associated with waterborne and foodborne outbreaks. However, viral hepatitis E tends to occur more often in young adults, many of whom are already immune to hepatitis A (Purcell, 1996). In contrast to hepatitis A, which rarely causes complications, hepatitis E tends to give rise to more prominent cholestasis and the infection can present as acute fulminating hepatitis, particularly in pregnant women, for whom case fatality rates as high as 20–40% have been recorded. Hepatitis E has an incubation period of 14–16 days, with a mean of 40 days (Reid & Dienstag, 1997), which is longer than that of hepatitis A. An exceptionally long viraemia is typical for HEV infection, generally lasting for as long as 6 weeks, and in some cases up to 16 weeks—again, substantially longer than for HAV. Patients generally excrete HEV for 1–2 weeks; in one case, however, the virus was excreted for more than 7 weeks, well after clinical and biochemical recovery (Clayson et al., 1995; Scharschmidt, 1995; Purcell, 1996).

Secondary transmission of HEV from cases to contacts has been reported but appears to be much less common than is true of HAV (Purcell, 1996). This seems surprising given that the duration of both faecal excretion and viraemia is substantially longer for HEV than for HAV. The low level of person-to-person spread probably implies that faecally polluted water plays a much more important role in the spread of HEV than of HAV. The explosive outbreaks of hepatitis E typically associated with waterborne transmission resemble the epidemiology of hepatitis A, and suggest that HEV may be as infective as HAV. The important role of water in the transmission of HEV may allow the virus to be transmitted from animals to humans via water resources polluted with animal wastes.

Although HEV is a single-stranded RNA virus with non-enveloped icosahedral capsid similar to that of HAV, the two viruses differ substantially at molecular level and HEV has been classified as a member of the family Caliciviridae.

There are indications of antigenic variation and possibly even differences in serotypes of HEV (Chauhan et al., 1994; Purcell, 1996; Schlauder et al., 1999; Tsarev et al., 1999). These strain variations seem to have implications for the molecular detection of the virus and for serological antibody assays (Mast et al., 1996; Ghabrah et al., 1998; Webber et al., 1998), and may even affect the immune status of patients. However, no individual has yet been reported as having contracted HEV infection more than once, which suggests that infection at any age generally results in lifelong immunity, as for HAV.

Despite some resemblances between HEV and HAV, there are also marked differences in their epidemiology. Both infections are primarily associated with poor standards of hygiene and sanitation, but the epidemiology of HEV seems also to include a geographical element (Grabow, Taylor & Webber, 1996). Clinical infections and outbreaks of hepatitis E have been recorded predominantly in countries such as Afghanistan, China, India, Myanmar, Nepal, and Pakistan, on the island of Borneo, and in parts of Central Asia; in Mexico; and in parts of Africa such as Algeria, Côte d'Ivoire, Egypt, Ethiopia, Somalia, and Sudan. The disease is endemic in many of these countries, and is the most common cause of acute hepatitis in adults in parts of India, other parts of Asia, and in Africa. Large outbreaks associated with sewage-contaminated drinking-water include one in 1954 involving approximately 40 000 cases in Delhi, India, one in 1986–1988 with more than 100 000 cases in the Xinjiang Uighar region of China, and one in 1991 with some 79 000 cases in Kanpur, India (Grabow et al., 1994; Scharschmidt, 1995).

Clinical cases, and outbreaks particularly, seem to occur rarely in parts of the world such as Japan, South Africa, the United Kingdom, North and South America, Australasia, and central Europe (Craske, 1992; Grabow, Taylor & Webber, 1996). Most cases that do occur in these parts of the world are imported. However, seroprevalence studies now reveal that the virus is actually present in many of these countries, and some 2–10% of the population may have antibodies, confirming exposure to the virus. Why there should be a relatively low incidence of clinical cases and outbreaks in certain parts of the world, despite the presence of the virus, is not yet fully understood (Grabow, Taylor & Webber, 1996). Answers to this and related questions are of fundamental importance because they may hold the key to methods for preventing worldwide spread of the virus and for control of the disease (Scharschmidt, 1995).

Since HEV is not readily detectable by conventional cell culture procedures, most of the initial work on the virus was confined to studies involving human volunteers, electron microscopy, immunological assays, and epidemiological data. The discovery that HEV causes infection in certain primates that resembles the infection in humans cast new light on the virus and its epidemiology (Bradley

et al., 1987). Research progress accelerated when molecular techniques became available (Favorov et al., 1992; Jothikumar et al., 1993). Subsequent studies revealed that at least some strains of the virus may also replicate in a variety of other animals, including laboratory rats (Maneerat et al., 1996; Meng, Guinet & Pillot, 1996), domestic pigs (Balayan et al., 1990), and rhesus monkeys (Sharma et al., 1990; Nanda et al., 1994).

The zoonotic nature of HEV was confirmed by Clayson et al. (1996) who detected the virus in a variety of wild and domestic animals (including cows, pigs, and goats); more recent findings even indicate the presence of HEV in rodents. In endemic areas the incidence of HEV in animals appears to correlate with that in humans. These findings suggest that animals may serve as a reservoir for HEV, and that many human infections may originate from water sources polluted by animal wastes (Kabrane-Lazizi et al., 1999; Wu et al., 2000). They also seem to be in agreement with the low level of human-to-human transmission mentioned earlier, and the detection of HEV in sewage in Barcelona, Spain—a part of the world where HEV is not endemic (Pina et al., 1998).

HEV thus seems to be unique as the only typical zoonotic member of the group of enteric viruses that are predominantly host-specific viruses infecting either humans or animals. If domestic and wild animals do indeed play an important role in the waterborne transmission of HEV, it underlines the need to protect water resources and supplies from pollution by animal wastes. In the past animal wastes were not considered to be of particular importance with regard to viruses: generally speaking, enteric viruses tend to be host-specific and there is scant evidence of human viral infections being contracted from water contaminated by animal wastes (Grabow, 1996).

Hepatitis F virus

The existence of HFV has not yet been conclusively proven: the name was proposed following reports of hepatitis cases associated with waterborne transmission of a virus distinguishable from HAV and HEV (Craske, 1992). Indications are that HFV is associated with sporadic cases in certain geographical areas, and not with outbreaks or epidemics (Sharma et al., 1990; Deka, Sharma & Mukerjee, 1994). Cases have been recorded in India, Italy, the United Kingdom, and the USA. In some of these areas clinical cases of HEV are virtually unknown and HAV is rare. Reasons for the apparent epidemiological pattern of HFV infection are not clear.

The virus seems to consist of a non-enveloped icosahedral particle, diameter 27–37 nm, containing double-stranded DNA. It has not yet been classified. Infection of rhesus monkeys has been reported, as has replication with cytopathogenic effect in the Hep-2 (human larynx carcinoma) cell line. Further details are primarily based on epidemiological data, electron microscopic detection of virus-like particles in patient stools, and clinical symptoms typical of enteric viral hepatitis in the absence of other causes of the disease. HFV does not

seem to be detectable by conventional routine cell culture techniques. Many questions about the virus remain to be answered, including those of infectivity, survival in the environment, and removal or inactivation by water treatment and disinfection processes.

Monitoring and assessment

Hepatitis viruses share the important characteristic of not readily causing a cytopathogenic effect in currently available cell culture systems. They are thus undetectable by conventional cell culture propagation procedures used for reoviruses, polioviruses, and some coxsackieviruses (Grabow et al., 1999). Well-established technology and expertise for direct monitoring and assessment of enteric hepatitis viruses in water and food are limited to HAV (Bosch et al., 1991a, 1991b; Hall & Sobsey, 1993; Tsai et al., 1994; Sobsey, Hall & Hazard, 1995). Detection of HEV in water or food has been reported only once using molecular techniques (Jothikumar et al., 1993), and there are no reports of HFV detection. Pina et al. (1998) amplified an HEV isolate from sewage in cynomolgus monkeys, but this technique would not be suitable for routine purposes. Observations that HEV may replicate in some cell cultures (Huang et al., 1992, 1995; Meng, Guinet & Pillot, 1996; Tam et al., 1996) could lead to the development of practical monitoring procedures, similar to those used for HAV, in which cell cultures are used to amplify at least the nucleic acid of the virus, which is then detected by molecular techniques (Grabow, 1997).

At this stage, even the technology for HAV is beyond the reach of many laboratories involved in water quality monitoring. Consequently, tests for hepatitis viruses are not currently recommended for conventional routine water quality monitoring. Analysis for hepatitis viruses is likely to be restricted largely to research purposes (Bosch et al., 1991a, 1991b; Grabow, 1997; Pina et al., 1998) until more practical and economical methods become available.

Assessment of the safety of water supplies with regard to hepatitis viruses—and many other viruses and other pathogens—therefore continues to depend largely upon indirect methods, such as the use of microbial indicators of faecal pollution. There is no doubt, however, that the commonly used methods have certain shortcomings with regard to indicating the presence of enteric viruses. For instance, outbreaks of hepatitis A have been associated with water supplies that conformed to generally accepted guidelines for indicators and treatment procedures (Hejkal et al., 1982; Bosch et al., 1991b; Grabow, 1997). Moreover, laboratory experiments have demonstrated that HAV, as well as at least some of the other enteric viruses, is more resistant to unfavourable conditions, including water treatment and disinfection processes, than commonly used indicators such as coliform bacteria (Grabow, 1997).

Water quality indicators should therefore be used with caution, and it may be necessary to employ combinations of indicators appropriately selected for various purposes (Grabow, 1996). Such combinations may have to include

indicators such as phages and *Clostridium perfringens*. It may also be advisable to supplement quality monitoring and assessment by meticulous sanitary surveys, and to base strategies on specifications for the quality of raw water sources and the efficiency of treatment and disinfection processes (Lloyd & Bartram, 1991; Regli et al., 1991; Sobsey et al., 1993; States & Sykora, 1995; Grabow, 1996; World Health Organization, 1997).

Indications are that HAV can successfully be recovered from water and food using techniques commonly applied to, for example, polioviruses and reoviruses. These include adsorption–elution procedures using positively or negatively charged membrane filters, followed by organic flocculation for secondary concentration (Sobsey, Oglesbee & Wait, 1985). HAV has been recovered from seeded drinking-water samples by means of ultrafiltration at an efficiency of recovery (EOR) of 100%, higher than that for polioviruses (Divizia, Santi & Pana, 1989). In a comparison of a number of recovery techniques for HAV, Bosch et al. (1991a) obtained the best results by adsorption–elution using glass powder of which the electrostatic charge had been changed to positive by treatment with polyethylenimine. For HAV in seeded 20-litre samples EOR was 100% for tap water, 94% for seawater and 61% for fresh water and sewage. HAV has also been recovered from sewage sludge (Graff, Ticehurst & Flehmig, 1993), shellfish meat suspensions (Deng, Day & Cliver, 1994; Jaykus, de Leon & Sobsey, 1996) and drinking-water supplies (Schwab, de Leon & Sobsey, 1996) by means of antigen capture techniques, using HAV-specific antibodies to recover the virus, but no specific details on EOR are available.

Jothikumar et al. (1993) successfully recovered HEV from raw and treated sewage by means of membrane filter adsorption–elution, followed by magnesium chloride precipitation. This procedure yielded a high EOR for enteroviruses, but the EOR for HEV has not been established. Sewage samples adjusted to pH 5.0 yielded positive PCR results for HEV, but samples adjusted to pH 3.5 failed to do so, which suggests that HEV is more sensitive to low pH levels than poliovirus, for example, which is recovered at this pH level in some routine techniques. Pina et al. (1998) were also successful in recovering HEV from sewage sample using ultracentrifugation of 40-ml samples; previously, the technique had achieved 70% recovery of seeded polioviruses.

Most of the above experiments on recovery of HAV were carried out using cell-culture-adapted strains of the virus, which can be titrated by conventional cell culture procedures or plaque assays. Detection of wild type HAV, however, requires different strategies, and the most feasible current approach is based on molecular techniques. HAV has been successfully detected in samples of wastewater, river water, and treated water by direct gene probe hybridization (Jiang et al., 1986; Dubrou et al., 1991) or PCR (Goswami, Koch & Cebula, 1993; Graff, Ticehurst & Flehmig, 1993; Tsai et al., 1993; Jaykus, de Leon & Sobsey, 1996; Schwab, de Leon & Sobsey, 1996). Goswami, Koch & Sebula (1993) claimed levels of sensitivity of 10 HAV RNA molecules in a reaction mixture of shellfish meat homogenate. Tsai et al. (1993) found their reverse transcriptase

(RT) PCR technique to be at least 500 times more sensitive for poliovirus than conventional cell culture detection, which suggests that the same procedure would also be extremely sensitive for HAV. In 1994 Tsai et al. reported the development of a triplex RT-PCR that simultaneously detected poliovirus, HAV, and rotavirus, radically reducing the time, cost, and labour involved in the monitoring of water supplies. Supplementation of molecular techniques by prior cell culture amplification of viral RNA substantially increases detection sensitivity, in addition to providing evidence that the detected HAV is viable (Dubrou et al., 1991; Shieh et al., 1991; Grabow, 1997).

Using a PCR procedure based on preparing HEV-specific cDNA by reverse transcription for amplification by PCR and detection by slot blot hybridization, Jothikumar et al. (1993) detected HEV recovered from sewage. The method followed by Pina et al. (1998) was similar, except that PCR products were analysed by agarose gel electrophoresis with ethidium bromide as the stain. Nested PCR products were characterized by determining nucleotide sequences (using an automated sequencer) and comparing them with HEV sequences in the GenBank and European Molecular Biology Library databases.

Information on the role of water and food in the transmission of HEV and HFV is primarily based on epidemiological data because practical methods for detection of these viruses are not yet available. Epidemiological data include indirect evidence obtained by seroprevalence studies: the presence of specific antibodies in individuals proves exposure to the virus, and a higher incidence of antibodies in communities exposed to contaminated water and inadequate sanitation indicates transmission by water and food (Grabow, Taylor & Webber, 1996; Tucker et al., 1996).

Control

The production of water supplies free of enteric hepatitis viruses, or with virus levels within tolerable limits, is possible and feasible yet is not always accomplished. The reasons for failure include cost, unavailability of expertise and facilities, and factors such as human negligence and error. Other contributing factors are the variable occurrence of viruses in polluted water sources (Halliday et al., 1991), and the exceptional resistance and high infectivity of the viruses—at least of HAV. Epidemiological statistics reveal that enteric hepatitis virus infections are much more frequently associated with waterborne transmission than infections with other enteric viruses (e.g. poliovirus, coxsackievirus, echovirus, adenovirus) (Grabow, 1997). In the laboratory HAV has been shown to be more resistant than faecal bacteria and certain phages to disinfecting agents such as chlorine, ozone, and hydrogen peroxide (Grabow et al., 1983; Rao et al., 1988; Mbithi, Springthorpe & Sattar, 1990; Hall & Sobsey 1993; Nasser et al., 1995). These findings are in agreement with reports on the detection of HAV in water that met generally accepted limits for faecal bacteria (Ford & Colwell, 1996; Grabow, 1996). The behaviour of HAV in the environment also differs from that of other

viruses and phages in respects such as survival in seawater and shellfish, resistance to ultraviolet light, and removal by filtration through soil columns (which reflects differences in adsorption properties) (Tsai et al., 1993; Callahan, Taylor & Sobsey, 1995; Lévêque et al., 1995; Sobsey, Hall & Hazard, 1995). The reasons for the successful transmission of enteric hepatitis viruses by water are neither fully understood nor quantifiable, and the roles of viral resistance and infectivity and of host susceptibility remain to be elucidated.

The only direct clue to the behaviour of HEV in the water environment is provided by detection of the virus in raw and treated wastewater, which suggests that the virus survives at least some wastewater treatment processes (Jothikumar et al., 1993). No such details are available on HFV. However, epidemiological evidence on their transmission by water and food leaves no doubt that all enteric hepatitis viruses must survive environmental conditions well enough to cause massive outbreaks of hepatitis A and E and sporadic case of hepatitis F.

The removal and inactivation of hepatitis viruses by individual treatment and disinfection processes commonly used in the preparation of drinking-water supplies have not yet been fully investigated. The principal reason for this is the lack of practical techniques for detecting viable naturally occurring hepatitis viruses. However, the behaviour of hepatitis viruses seems to resemble that of other viruses and phages sufficiently for data on polioviruses and reoviruses, for example, and on phages like somatic and F-RNA coliphages to provide at least an indication of what can be expected for hepatitis viruses.

In laboratory experiments on flocculation-enhanced rapid sand filtration, for instance, Nasser et al. (1995) found turbidity to be reduced by 99%, a vaccine strain of poliovirus by 80%, and a cell-culture-adapted strain of HAV and F-RNA phage MS2 both by 93%. Vaccine poliovirus was reduced by as much as 99% in units using aluminium sulfate, ferric sulfate, or ferric chloride coagulation and rapid sand filtration (Bitton, 1980). In a plant for the direct reclamation of drinking-water from wastewater, ferric chloride clarification followed by rapid sand filtration reduced naturally occurring cytopathogenic viruses by 88–99%, depending on the operating conditions (Grabow, 1990). Water-softening using a lime-soda ash process reduced vaccine poliovirus by more than 99% (Bitton, 1980). In laboratory experiments, activated carbon filters removed 75–82% of vaccine poliovirus and 53–86% of coliphage T4, depending on the load of seed virus and phage (Bitton, 1980); these results agreed with data recorded on a water reclamation plant (Grabow, 1990).

For groundwater, available evidence indicates reasonable similarities in the behaviour of various enteric viruses and phages such as MS-2 (Yates, Gerber & Kelley, 1985; Sobsey, Hall & Hazard, 1995), and there is no reason to believe that the behaviour of enteric hepatitis viruses is significantly different. It therefore seems that existing recommendations for the utilization and treatment of groundwater (World Health Organization, 1996) can be trusted to cover hepatitis viruses.

Disinfection processes have been investigated in more detail. Laboratory experiments using a variety of viruses and phages, and detailed analysis of disinfection processes in water-treatment plants confirm that viruses and phages are at least 99% inactivated by recommended disinfection methods (Bitton, 1980; Grabow, 1990). Recommended conditions of disinfection include those made by the World Health Organization (1993, 1996) for conventional water treatment purposes, which specify a free chlorine residual of at least 0.5 mg/litre for 30 minutes at pH < 8.0, with mean turbidity not exceeding 1 nephelometric turbidity unit (NTU). Free chlorine residuals of 1 mg/litre and an exposure time of 60 minutes have been recommended for the disinfection of drinking-water directly reclaimed from wastewater (Grabow, 1990). In laboratory experiments, cell-culture-adapted strains of HAV were unable to survive these conditions of disinfection; the rate of inactivation resembled that of vaccine polioviruses and F-RNA phages (Grabow et al., 1983; Sobsey, 1989). Similarly efficient inactivation of cell-culture-adapted strains of HAV has been reported for other oxidizing agents such as ozone and ozone-hydrogen peroxide (Hall & Sobsey, 1993), and for other commonly used disinfection processes such as ultraviolet light irradiation (Wiedenmann et al., 1993).

Generally accepted goals for the overall efficiency of water-treatment plants include those of the United States Environmental Protection Agency, which specify a minimum 4 log (4 orders of magnitude) reduction in virus numbers in water sources of acceptable quality (Rose & Gerba, 1991). Evidence has been presented that conventional water-treatment plants operated in accordance with design specifications are capable of this level of efficiency (Bitton, 1980; Grabow, 1990; Ford & Colwell, 1996; World Health Organization, 1996). However, design specifications are usually based on the optimal functioning of all barriers in the system, which implies that any breakdown, malfunction, or suboptimal operation will impair the efficiency of the system. Highly resistant pathogens like viruses are the most likely to survive in such a situation. If loss of efficiency coincides with exceptionally high numbers of viruses in the raw water intake (because of seasonal fluctuations or a disease outbreak), there is a risk of infection (Halliday et al., 1991). Events of this kind probably explain why viral hepatitis and certain other diseases are often associated with drinking-water supplies from plants apparently operating in accordance with accepted specifications (Bosch et al., 1991b; Ford & Colwell, 1996; Grabow, 1996, 1997).

The early detection of failures in water-treatment plants depends heavily upon the regular monitoring of quality. Unfortunately, considerations of cost often mean that monitoring is restricted to inexpensive, simple, and rapid tests, such those for coliform bacteria. These faecal bacteria are not reliable indicators of the presence of viruses, as evidenced by the frequency with which hepatitis and other viral infections are associated with drinking-water supplies that meet generally accepted criteria for faecal coliforms (Hejkal et al., 1982; Bosch et al., 1991b; Ford & Colwell, 1996; Grabow, 1996; Payment, 1997).

Despite exceptional resistance, there is no evidence that hepatitis or any other viruses can survive recommended conditions of water treatment and disinfection

(World Health Organization, 1993, 1996). However, the production of safe drinking-water supplies requires rigorous and failsafe application of these specifications. Important aspects of the recommendations include the utilization of treatment processes appropriate according to source water quality, subject to periodic verification and continuous monitoring of process efficiency (World Health Organization, 1993, 1997). This will normally require frequent testing of physico-chemical indicators of treatment and disinfection processes. Shortcomings of conventional coliform indicators for monitoring the microbiological quality of water and the efficiency of treatment processes could be supplemented by using appropriately selected combinations of additional indicators, which could include highly resistant organisms such as *Clostridium perfringens*, phages, and heterotrophic plate counts (Ford & Colwell, 1996; Grabow, 1996). This would apply in particular to potentially high-risk situations such as the production of drinking-water from heavily polluted source water (Grabow, 1990). Ideally, of course, monitoring of treatment plants and drinking-water supplies should include tests for viruses, but this is not yet within the technical or financial reach of many water suppliers.

Recently introduced vaccines may offer protection against waterborne HAV. However, no vaccines are available for HEV and HFV, and there is no indication of any becoming available in the foreseeable future. Similarly, immunoglobulin preparations are available for temporary protection against HAV, but no such preparations are available for protection against HEV and HFV. There is no chemotherapeutic treatment for infections caused by any of these viruses—only supportive treatment. The benefits of HAV immunization are not altogether clear. Prevention of infection in endemic areas would require immunization of very young children. However, the vaccines consist of inactivated HAV and, unlike natural infection, do not confer lifelong immunity. Early immunization may simply shift susceptibility from childhood to later in life, when the impact of infection is more severe. This, like the similar situation that arose with poliovirus (Iwarson, 1992), is clearly undesirable. Moreover, the benefit for the growing number of immunocompromised individuals remains uncertain. For individuals in specific situations, such as health care workers in critical service areas, sewage workers, and military personnel deployed on short notice to high-risk areas, HAV immunization may indeed offer significant advantages. However, immunization does not seem to offer a practical or universal alternative to control based on prevention, i.e. safe water and food, and good standards of hygiene and sanitation.

Conclusions and recommendations

Health risk assessment

The enteric hepatitis viruses continue to be responsible for a significant disease burden—both through outbreaks of disease and through endemicity. They are all transmitted by the faecal–oral route, typically through drinking-water and

food in which they appear to be highly infectious. The only known reservoir of HAV and HFV is the human population. By contrast, recent evidence indicates that HEV is much more zoonotic than was previously thought, and a variety of domestic and wild animals may serve as reservoirs for the virus. This implies that faecal pollution of both human and animal origin may pose a risk of HEV infection.

The HAV vaccines that have been developed may be used to protect individuals in high-risk situations, but the principal barriers to transmission of enteric hepatitis virus are safe drinking-water supplies and good standards of sanitation and of personal and food hygiene. The relative importance of environmental transmission of enteric hepatitis viruses is likely to increase as demands on limited water resources encourage water reuse, as improvements in basic water treatment tend to eliminate the more susceptible pathogens (such as *Salmonella* and *Vibrio*), and as the age of initial exposure to the viruses increases.

Risk management

Risk management includes specifications for the quality of raw water sources and the efficiency of treatment and disinfection processes outlined earlier. Use of appropriate combinations of indicators for quality monitoring and assessment, and programmed sanitary surveys for pollution of human and animal origin should also be integral components of risk-management strategy.

Enteric hepatitis viruses are highly infectious. Although individual risk may be minimal in areas of low endemicity, there remains the risk of sporadic introduction of the viruses by symptomatic or asymptomatic individuals, especially returning travellers and their immediate contacts. Effective control where the water supply is derived from faecally contaminated sources requires multiple treatment barriers, including chemical disinfection. In areas of high endemicity and/or low water pressure or high leakage rates in distribution systems, the use of a residual disinfectant is required, with safe concentrations maintained throughout the distribution system.

Specific analysis of water for hepatitis viruses would be carried out only in exceptional circumstances—predominantly for research intended to elucidate how the viruses could have survived treatment and disinfection processes. Comprehensive quality surveillance programmes, designed to detect the earliest possible indications of quality fluctuations in raw water sources, treatment failure, doubtful final quality of the water, or relevant incidences of disease in communities concerned, are valuable tools for risk management (Grabow, 1996).

Research priorities

In view of the shortcomings of indicator systems and indirect quality assessment methods, development of practical methods for direct detection and monitoring of viruses remains a high priority (Sobsey et al., 1993; Ford & Colwell, 1996).

This is especially important for research on the behaviour of viruses in treatment and disinfection processes. The many questions about the epidemiology of enteric hepatitis viruses, such as the apparent geographical distribution of HEV and HFV, should also be addressed, since they are crucial to improved risk management and to the control of local waterborne transmission and worldwide dissemination of these viruses. Technological progress, particularly the development of molecular techniques, suggests that important new tools and information may soon become available.

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Legionella¹

Description

Species of the genus *Legionella* are Gram-negative, non-spore-forming, rod-shaped, aerobic bacteria. They contain branched-chain fatty acids, have a non-fermentative metabolism, and require L-cysteine and iron salts for growth. They have been placed in the family Legionellaceae, which contains the single genus *Legionella*; there are at least 42 species, which are listed in Table 5 (Drozanski, 1991; Adeleke et al., 1996; Hookey et al., 1996; Fry & Harrison, 1998; Riffard et al., 1998). The type species is *L. pneumophila*. Two other genera have been proposed but have not received general recognition (Garrity, Brown & Vickers, 1980): *Fluoribacter* for the blue-white fluorescing species such as *L. bozemanii* and *L. dumoffii*, and *Tatlockia* for the species *L. micdadei*. Some species of *Legionella* can be further differentiated into serotypes, of which there are at least 15 for *L. pneumophila* but so far no more than two for any other species.

Free-living legionellae are rod-shaped, 0.3–0.9 µm wide and approximately 1.3 µm long. They will grow to 2–6 µm in vitro, but can form filaments 20 µm or more in length. Although they are Gram-negative, legionellae actually stain poorly in the Gram procedure and by other similar staining methods, particularly in infected tissues. This has been attributed to the presence of the branched-chain fatty acids that are a major component of the cell walls. Other staining methods have been described, such as the silver impregnation method of Dieterle (Dieterle, 1927); the most effective methods include antibody-coupled fluorescent dyes and immunoperoxidase staining.

The legionellae are usually motile by means of one or more polar or subpolar flagellae. The cell wall consists of a cytoplasmic membrane on the inner

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Table 5. Legionella species and serogroups and their association with disease

Legionella species	Serogroups	Pathogenicity for humans
<i>L. adelaidensis</i>		
<i>L. anisa</i>		+
<i>L. birminghamensis</i>		+
<i>L. bozemanii</i>	2	+
<i>L. brunensis</i>		
<i>L. cherrii</i>		
<i>L. cincinnatiensis</i>		+
<i>L. donaldsonii</i> ^a		
<i>L. dumoffii</i>		+
<i>L. erythra</i>	2	
<i>L. fairfieldensis</i>		
<i>L. feeleii</i>	2	+
<i>L. gesstiana</i>		
<i>L. gormanii</i>		+
<i>L. gratiana</i>		
<i>L. hackelae</i>	2	+
<i>L. israelensis</i>		+
<i>L. jamestowniensis</i>		
<i>L. jordanis</i>		+
<i>L. lansingensis</i>		
<i>L. londiniensis</i>	2	
<i>L. longbeachae</i>	2	+
<i>L. lytica</i> ^b		
<i>L. maceachernii</i>		+
<i>L. micdadei</i>		+
<i>L. moravica</i>		
<i>L. nautarum</i>		
<i>L. oakridgensis</i>		+
<i>L. parisiensis</i>		+
<i>L. pneumophila</i>	15	+
<i>L. quateirensis</i>		
<i>L. quinlivanii</i>	2	
<i>L. rubrilucens</i>		
<i>L. saithelensi</i>	2	+
<i>L. santicrucis</i>		+
<i>L. shakespearei</i>		
<i>L. spiritensis</i>		
<i>L. steigerwaltii</i>		
<i>L. tucsonensis</i>		+
<i>L. wadsworthii</i>		+
<i>L. waltersii</i> ^c		
<i>L. worsleiensis</i>		

^a Adeleke et al , 1996.^b Drozanski, 1991.^c Hookey et al , 1996

surface, a thin peptidoglycan layer, and an outer membrane that contains the heat-stable lipopolysaccharides (LPS) with species- and serogroup-specific O antigens. There is no definitive evidence of a capsule.

The optimal temperature for in-vitro growth is 36 °C (limits 15–43 °C), with a generation time of 99 minutes under optimal conditions (Brenner, Fealey & Weaver, 1984; Brenner, 1986; Fallon, 1990; States et al., 1993). In the natural habitat—fresh water and soil—growth requires the presence of other bacteria or of protozoa, which are considered to be the natural hosts of legionellae (Rowbotham, 1980; Tison et al., 1980; Wadowsky & Lee, 1985; Fields et al., 1993).

Pathogenicity for humans

Legionellae were first detected in 1976 after a particularly notable outbreak of pneumonia in a hotel on the occasion of a United States army veterans' meeting (Fraser et al., 1977). Since that time, it has been established that these organisms are an important cause of pneumonia, both community-acquired (1–15%) (Lieberman et al., 1996; Butler & Breiman, 1998) and hospital-acquired (up to 50%) (Butler & Breiman, 1998). To date, disease due to *Legionella* has been detected almost exclusively in humans, but some animals (e.g. guinea-pigs, rats, mice, marmosets, and monkeys) are susceptible to experimental infection. One case of *Legionella* pneumonia has also been reported in a calf (Fabbi et al., 1998). Evidence of past infection can also be found in other animal species, including wild animals, but no animal reservoir of the bacteria or transmission between animals has been demonstrated (Collins, Cho & Reif, 1982; Boldur et al., 1987).

Two kinds of disease are observed in humans. Legionnaires disease is a severe pneumonia (incubation time 2–10 days); mortality is about 15% and *Legionella* may be detected in sputum and tissues. Pontiac fever is a febrile illness of 2–6 days' duration, with an incubation time of 1–7 (normally 3) days; it is non-pneumonic (cough is observed in about 50% of cases) and self-limiting, and accompanied by headache and myalgia. Bacteria are not detectable in body fluids or tissues nor are bacterial antigens found in urine, but blood antibodies are elevated (Glick et al., 1978; Fallon et al., 1993).

Legionnaires disease is commonly accompanied by extrapulmonary manifestations, such as renal failure, encephalopathy, and pericarditis (Oredugba et al., 1980; Posner et al., 1980; Riggs et al., 1982; Mayock, Skale & Kohler, 1983; Johnson, Raff & Van-Arsdall, 1984; Nelson et al., 1984). Lung abscesses, other local infections, and wound infections involving *L. pneumophila* and *L. dumoffi* have also been reported (Arnow, Boyko & Friedman, 1983; Bauling, Weil & Schroter, 1985; Lowry et al., 1991).

L. pneumophila serogroup 1 is most commonly isolated from patients (58% of isolates in England and Wales, 71.5% in the USA) (Joseph et al., 1994; Marston, Lipman & Breiman, 1994), followed by *L. pneumophila* serogroup 6 (Tang & Krishnan, 1993). Other serogroups of *L. pneumophila* and another 19 species of *Legionella* are associated to a varying degree with human disease. In

the USA, *L. micdadei* is the second most frequent cause of Legionnaires disease and has also been repeatedly identified as the causative agent of Pontiac fever (Goldberg et al., 1989; Lutichau et al., 1998). In Australia, *L. longbeachae* seems to be an important cause of Legionnaires disease (Steele, Lanser & Sangster, 1990). Between 9.3% and 29.0% of infections are caused by species other than *L. pneumophila* (Tang & Krishnan, 1993; Joseph et al., 1994; Marston, Lipman & Breiman, 1994), but for most of these there are neither properly validated serological tests nor optimized isolation media (Edelstein, 1993).

Pathogenicity of *Legionella* in humans is largely dependent on host susceptibility. Children and young people are rarely affected, while immunocompromised individuals—especially transplant recipients—are at very high risk of disease. However, since any population may exhibit both extremes of susceptibility, even people considered to be “fit and well” may become ill (World Health Organization, 1990). Lieberman et al. (1996) observed that 39 out of 56 patients with community-acquired *Legionella* pneumonia had no chronic comorbidity, although coinfection with another microorganism was frequent. Smoking and alcoholism are commonly acknowledged to be predisposing factors, and infection is more common in males than females and in people over 40 years of age (World Health Organization, 1990; Butler & Breiman, 1998). Individual risk factors also include working more than 40 hours per week and spending nights away from home (Straus et al., 1996).

Individuals with terminal renal insufficiency or blood malignancies, people receiving steroid treatments, and severely immunocompromised individuals (including those with HIV/AIDS) are at significant risk for acquiring Legionnaires disease (Marston, Lipman & Breiman, 1994). Patients with chronic lung disease, cirrhosis of the liver, or diabetes are also at risk, though to a slightly lesser extent. An indwelling nasogastric tube is a further independent risk factor for nosocomial Legionnaires disease (Marrie et al., 1991; Blatt et al., 1993). Pontiac fever, by contrast, affects children and healthy adults just as frequently as immunocompromised individuals (Goldberg et al., 1989).

During an outbreak of disease, exposed populations frequently show elevated serum antibody levels but no symptoms of disease. The same is true of people working in high-risk areas. It has been reported that 62 out of 143 (43.4%) healthy people exposed to a contaminated environment had positive antibody titres against distinct serogroups (Paszko-Kolva et al., 1993).

Virulence factors

Legionellae are intracellular pathogens of macrophages, by which they are phagocytosed in a process involving the complement fragment C3 and the monocyte complement receptors CR1 and CR3. Both virulent and non-virulent strains are phagocytosed, remaining intact inside the phagocytes. Virulent strains can multiply inside the phagocytes and are able to inhibit the fusion of phagosomes with lysosomes; non-virulent strains do not multiply (Horwitz, 1993). Only two prod-

ucts of *Legionella* have so far been shown to be associated with virulence (Fields, 1996)—the 24-kDa protein, macrophage infectivity potentiator, thought to be conserved throughout the genus (Ciancotto et al., 1989, 1990; Riffard et al., 1996), and the 113-kDa integral protein of the cytoplasmic membrane, which is the product of the *dotA* gene (defect in organelle tracking) (Berger & Isberg, 1993; Berger, Merriam & Isberg, 1994; Roy & Isberg, 1997). Helbig et al. (1995) have proposed that differences in the virulence of *Legionella* species or serogroups are associated with differences of epitopes of the LPS. Within *L. pneumophila* serogroup 1, the strains most commonly associated with disease in humans share a common epitope, as revealed by monoclonal subtyping (Watkins et al., 1985; Ehret, von Specht & Ruckdeschel, 1986; Dournon et al., 1988). Aerosol survival (Dennis & Lee, 1988), growth temperature (Mauchline et al., 1994), the possession of tissue-destructive protease (Baskerville et al., 1986), and the expression of flagellae (Bosshardt, Benson & Fields, 1997) may also be important virulence factors.

The host defence against *Legionella* relies principally on cell-mediated immune mechanisms. One protein produced by *L. pneumophila*, the major secretory protein (MSP, 39 kDa), is able to induce protective cell-mediated immunity without being a virulence factor (Blander & Horowitz, 1991). Circulating antibodies are produced during infection with *L. pneumophila* in humans, but they do not seem to be protective and antibody titres rise only slowly; 30% of patients do not produce antibodies detectable by immunofluorescence-coupled antigens up to 4 weeks after infection. Rising levels of serum antibodies, however, are of great diagnostic and epidemiological value. No vaccine has so far been tested in humans.

Dose–response relationship; animal studies

Inoculation of guinea-pigs with material from the lungs of infected individuals resulted in the first isolation of *L. pneumophila* in 1977 (McDade et al., 1977). Since that time, guinea-pigs have been used repeatedly for experimental infection and have proved susceptible to infection by inhalation, although aerosol infection is in fact very difficult to achieve (Yu, personal communication). The lethal dose varies from 2400 to 100 000 viable bacteria, but infection can be initiated by as few as 130 organisms. Infections have also been induced in monkeys, rats, and mice, although mice seem to be somewhat resistant, at least in terms of mortality (Baskerville et al., 1981; Collins, 1986). The susceptibility of the A/J mouse strain is due to a single recessive gene conferring permissiveness on A/J macrophages (Beckers et al., 1995). Suckling CD1 mice have been shown to be susceptible to infection and seem to provide a promising animal model for studies of *L. pneumophila* virulence (Castellani Pastoris et al., 1997).

The infective dose for humans can be assumed to be low—possible even a single organism—since *Legionella* infections have frequently been traced to contaminated aerosols generated at distances of up to 3.2 km (Addiss et al., 1989).

Given the frequency of *L. pneumophila* in human surroundings, the virulence of the organism, and the fact that the infective dose is so low, a much larger number of infections would be expected than is actually the case. It therefore follows that there must be other, as yet unknown, determinants of infection. Infectivity may be substantially enhanced if amoebae are inhaled or aspirated (Brieland et al., 1996). Vacuoles in infected amoebae may contain many hundreds of *Legionella* cells which, when liberated, provide a large inoculum in a restricted area of the respiratory tract (Rowbotham, 1986; O'Brien & Bhopal, 1993; Berk et al., 1998).

Mode of transmission

Inhalation of airborne droplets or droplet nuclei containing legionellae is generally thought to be the commonest mode of transmission. The aerosols may be generated by mechanical devices (e.g. cooling towers of air-conditioning systems) or by the use of potable water, especially from domestic hot-water installations (e.g. showers) (Breiman et al., 1990). In one cluster of infections, *L. longbeachae* was isolated from potting mixes and the soil of potted plants in the vicinity of patients (Steele et al., 1990, 1993, 1996). Three cases of Legionnaires disease due to *L. pneumophila* were reported following the flooding of the basement of a bar; bacteria were isolated from the sump water (Kool et al., 1998).

Aerosol formation is deemed necessary to cause pneumonic disease, but aspiration following ingestion of contaminated water, ice, and food has also been implicated as the route of infection in some cases (Marrie et al., 1991; Blatt et al., 1993; Venezia et al., 1994; Graman, Quinlan & Rank, 1997). Some authors believe aspiration to be the major mode of transmission (Yu, 1993). Sporadic cases in hospitals have arisen from use of the taps in wash-basins. Even when it is possible to demonstrate that the disease strain and the strain colonizing a plumbing system are identical, the exact route of transmission sometimes remains a matter of speculation. There is no evidence of person-to-person transmission (Fraser, 1977; Yu, 1983).

Outbreaks and single cases of Legionnaires disease have been traced to the cooling towers and evaporative condensers of air-conditioning systems, decorative fountains, ultrasonic nebulizers, room humidifiers, hot whirlpool and spa baths, hot water from taps and showers, and medical devices containing water (e.g. respiratory care devices) (Butler & Breiman, 1998). Of 20 hospital outbreaks of Legionnaires disease in England and Wales between 1980 and 1992, 19 were attributed to *Legionella*-contaminated potable water systems (Joseph et al., 1994). The hot-water plumbing systems of many hospitals are contaminated and colonized by legionellae. The same strain may be identified over extended periods at particular sampling points (Chang et al., 1996), but different strains may colonize different parts of the same building (Marrie et al., 1992).

Disease occurrence: outbreaks, sporadic cases, and prospective studies

Outbreaks

Since the 1976 outbreak in Philadelphia led to the detection and description of the family Legionellaceae, many outbreaks—a number of them spectacular, but most on a smaller scale—have been reported, frequently involving hospitals. Infections have often been traced to colonized parts of air-conditioning plants (Dondero et al., 1980; Addiss et al., 1989; O'Mahoney et al., 1990; Watson et al., 1994), but most outbreaks and recurrent single cases in hospitals are associated with contaminated potable water and hot-water systems (Joseph et al., 1994). Decontamination of colonized installations has been shown to interrupt outbreaks and prevent recurrence of sporadic cases. In two prospective studies in hospitals, the frequency with which *L. pneumophila* was isolated from patients with pneumonia was reduced from 16.3% to 0.1% over a 6-year period and from immunocompromised patients from 76% to 0.8% over a 10-year period (Grosserode et al., 1993; Junge-Mathys & Mathys, 1994). Measures used to achieve this included decontamination of the plumbing systems, monitoring of *Legionella* in the water, examination of all clinical specimens for signs of *Legionella* infection, use of sterilized water for all applications in high-risk patients, and ensuring that all patients and clinical staff were adequately informed of the risks of infection, especially with respect to the use of hot water in high-risk wards.

Travel-associated Legionnaires disease

Legionnaires disease is often associated with travel and with staying in hotels—as was the case in the 1976 outbreak in Philadelphia. A study carried out in Ohio (Straus et al., 1996) on domestic acquisition of Legionnaires disease identified nights spent away from home as a risk factor. In England and Wales, 56% of the 160 cases reported in 1995 occurred in travellers (Newton et al., 1996), and in 1997 the same was true for 114 of the 226 reported cases (Joseph et al., 1998). Among 52 Finnish patients with Legionnaires disease, 76% of those who were not immunosuppressed and had no underlying disease ($n = 17$) had made recent journeys (Skogberg et al., 1994). Small clusters of cases have repeatedly been reported among tourists staying at certain hotels in holiday resorts, especially in the Mediterranean region: 55% of 119 hotels in various European countries had legionellae in their water distribution systems and 73% had amoebae (Starlinger & Tiefenbrunner, 1996).

Outbreaks reported among passengers on cruise ships have been traced to contaminated water in whirlpool baths (Jernigan, 1996) or to drinking-water (Castellani Pastoris et al., 1999). Gerchikova et al. (1990) have found immunological evidence of increased exposure among railway conductors, subway personnel, and railroad construction workers, and have isolated two strains of

L. pneumophila from water samples taken from railway dining cars. Water pipes and reservoirs on ships, railway carriages, and the like are often subject to warming and are not easily emptied for cleaning. Chlorine decay and bacterial growth are thus more likely in the water they contain.

The link between travel and Legionnaires disease was discussed at a WHO meeting in 1989 (World Health Organization, 1990). A surveillance scheme for travel-associated Legionnaires disease, instituted by the European Working Group on *Legionella* Infection, coordinated by the Public Health Laboratory Service in London, England, and monitored by WHO, has led to the detection of many cases and improved disease prevention.

Sporadic community-acquired infections

In a prospective study in two counties in Ohio, USA, Marston et al. (1997) showed that most cases of pneumonia caused by *Legionella* are community-acquired and sporadic. The annual incidence (with definite diagnosis) was calculated to be 7.0/100 000 adults—approximately 10 times the number of cases reported to health authorities. Community-acquired infections may be caused partly by cooling towers and other aerosol-producing devices, but certain features of domestic plumbing and potable-water supply and water-heating systems have also been shown to be associated with Legionnaires disease and must therefore also be considered as a sources of legionellae (Aldea et al., 1992; Straus et al., 1996).

Plumbing systems in residential premises—particularly one-family houses (Tiefenbrunner et al., 1993)—are less frequently colonized than those in hospitals. However, investigations in different cities in Finland, Germany, and Spain have shown that apartment blocks may be as heavily contaminated as hospitals (Aldea et al., 1992; Lück et al., 1993; Zacheus & Martikainen, 1994). The observed differences may be due to the size of water heaters, the extent of the hot-water installations, and other details of the heating (central versus point-of-use, electric versus gas or oil) and distribution systems (Alary & Joly, 1991).

Monitoring and assessment

Examination of clinical specimens

No specific clinical symptoms of *Legionella* infections distinguish them from pneumonia or localized infections of other origins, and many community-acquired infections will be treated without diagnosis. Definitive diagnosis of a *Legionella* infection relies on the following features:

- increasing serum concentrations of antibodies;
- detection of antigens in the urine;
- detection of bacteria in lung tissue, or in sputum or other secretions, by direct immunofluorescence microscopy;

- culture of *Legionella* from respiratory secretions, bronchoalveolar lavage fluid, pleural fluid;
- detection of *Legionella* nucleic acid by DNA probes or by polymerase chain reaction (PCR).

Up to 4 weeks after infection, 30% of patients do not develop antibodies detectable by indirect immunofluorescence assay (IFA). An acute-phase antibody titre of 1:256 did not discriminate between cases of *Legionella* infection and non-cases, while a positive urine antigen assay was found in 55.9% of cases compared with <1% of non-cases (Plouffe et al., 1995). A fourfold increase in IgG and IgM titres is considered to be a reliable sign of infection, and detection of *Legionella* antigen is a fairly sensitive (70%) and highly specific (>99%) method for diagnosis of *L. pneumophila* serogroup 1 infection (Plouffe et al., 1995). Urinary antigen test results will remain positive for several weeks after the onset of infection (Stout & Yu, 1997). However, infections with non-serogroup 1 *L. pneumophila* will be missed unless test kits containing antibodies against other *Legionella* serogroups and species are available. Urinary tests to detect infection with other serogroups and species are being developed.

Detection of bacteria in lung tissue and sputum, by direct immunofluorescence as well as by DNA hybridization and PCR is no more successful than the examination of serum for antibodies or of urine for antigen—70% of cases, at best, are detected. Clearly, a positive bacterial culture is the most convincing evidence of infection, but only 9% of 160 cases reported in England and Wales in 1995 were diagnosed by culture (Newton et al., 1996).

To summarize, there is no single laboratory test currently available that will detect all infections caused by *L. pneumophila* or other *Legionella* species (Edelstein, 1993).

Analytical methods for environmental samples

A standard procedure for the isolation, culture, and identification of *Legionella* has been prepared by the International Organization for Standardization (1998). High-yield solid and liquid culture media are commercially available; these are generally optimized for *L. pneumophila*. Recovery rates using these media, and using sample preparation procedures, have yet to be fully evaluated for other *Legionella* species. In dealing with *Legionella* species other than *L. pneumophila*, therefore, the recovery rate should be determined. Steinert et al. (1997) have shown that legionellae may enter a viable but non-culturable state, but become culturable again by cocultivation with axenic *Acanthamoeba castellanii*. Swab specimens from a faucet have been shown to yield 10 times as many legionellae as a 250-ml water sample taken from the same faucet (Ta et al., 1995), reflecting the prevalence of the organisms in biofilms.

Environmental samples frequently need to be concentrated or diluted to give optimal results on solid media. Moreover, background bacteria must be eliminated before, or suppressed during, primary culture. Legionellae and background

bacteria can be concentrated by centrifugation (e.g. 6000g for 10 minutes at about 20°C) or by membrane filtration. Numbers of other bacterial species present in the sample can be reduced by heat treatment (50 ± 1°C for 30 ± 2 minutes, or 55°C for 15 minutes) or by acid treatment (3 minutes at pH 2.2). The material is then streaked, or the filter is transferred, onto buffered charcoal–yeast extract (BCYE) agar (Edelstein, 1981), with or without selective supplement. Various improved media for different purposes have been proposed more recently, as has incubation under 2–5% carbon dioxide. A medium containing dyes (bromocresol blue and bromocresol purple), vancomycin, and polymyxin B (DGVP) gave optimal results in a comparative study (Ta et al., 1995).

Plates are incubated at 36 ± 1°C for up to 10 days and examined every 2 or 3 days. Presumptive *Legionella* colonies are examined for their L-cysteine requirement by streaking them onto cysteine-free BCYE agar or other appropriate media, e.g. sheep blood agar, with subsequent incubation. Confirmation of *Legionella*, and species and serotype identification are done using commercially available antisera, preferably by direct immunofluorescence. Commercially available latex agglutination kits may also be used. A more rapid procedure has been proposed to replace examination for L-cysteine requirements, namely a colony blot assay using a genus-specific monoclonal antibody coupled with a chromogenic reagent (Obst, 1996). PCR procedures have also been developed. The DNA-sequence information of the ribosomal 23S–5S spacer region was used to develop a genus- and species-specific detection and identification system for all legionellae, using PCR and reverse dot-blotting (Robinson et al., 1996).

Both environmental strains and clinical isolates can be successfully subtyped by molecular techniques such as ribotyping, macrorestriction analysis by pulsed-field gel electrophoresis, or PCR-based methods (Schoonmaker & Kondracki, 1993; Pruckler et al., 1995; Van Belkum et al., 1996). These yield valuable information on the sources and epidemiology of infections. However, the results of subtyping alone, in the absence of epidemiological data, cannot reliably implicate a source because the distribution of the various subtypes in the environment is unknown.

Control

Occurrence, transport, and survival in the environment and in source waters

The legionellae have been found in natural freshwater systems, including thermal waters, all over the world and are considered to be part of the natural freshwater microbial ecosystem (Fliermans et al., 1981; Verissimo et al., 1991). The organisms have also been found in sewage-contaminated coastal waters of Puerto Rico (Ortiz-Roque & Hazen, 1987), in well material down to a depth of 1170 metres (Fliermans, 1996), and in low concentrations in groundwater (Frahm & Obst, 1994; Lye et al., 1997). Some outbreaks have been associated with soil and

excavation activities. *L. longbeachae*, *L. bozemanii*, and *L. dumoffi* have all been isolated from potting mixes made from composted wood wastes (Hughes & Steele, 1994; Steele & McLennan, 1996).

A characteristic feature of legionellae is their ability to multiply inside protozoa (Rowbotham, 1980). Protozoa that support the growth of legionellae include species of *Acanthamoeba*, *Hartmanella*, *Naegleria*, *Echinamoeba*, *Vahlkampfia*, and *Tetrahymena* (Fields, 1993). Indeed, it has been suggested that environmental growth of legionellae in the absence of protozoa has not been demonstrated, and that protozoa are the natural reservoir for these organisms in the environment (Fields, 1993). However, association with cyanobacteria of the genera *Fischerella*, *Phormidium*, and *Oscillatoria* also promotes relatively rapid growth of *L. pneumophila* (Tison et al., 1980), and cocultivation with some bacteria has been demonstrated in vitro (Wadowsky & Yee, 1985).

Legionellae will not grow in sterilized samples of the water from which they have been isolated. It follows from this that they are part of a microbial ecosystem in which they are both nourished and protected from physical removal by the water current and from antimicrobial agents. They are detected in significant numbers only after other microorganisms have colonized sediments, soil, or biofilms. Growth of other *Legionella*-like organisms in amoebae has been described repeatedly, and—on the basis of 16S rRNA similarity—it has been proposed that these organisms are indeed members of the genus *Legionella* (Rowbotham, 1993; Adeleke et al., 1996).

With the exception of thermal waters and water in tropical regions, legionellae are found in only low concentrations in natural environments (≤ 1 cfu/ml in groundwater); this is to be expected from the low replication rates at temperatures below 25 °C. The organisms will be introduced from surface water, soil, and subsoil into water used as the source for preparation of drinking-water and other purposes.

Effects of drinking-water treatment

Storage of raw water in reservoirs will not necessarily reduce numbers of *Legionella*; at elevated temperatures there may even be growth of the organisms. However, as for other bacteria, *Legionella* concentrations can be reduced by coagulation, flocculation, and sedimentation. Growth of *Legionella* may well occur inside filters used for drinking-water preparation (such as granular activated carbon filters) if there is microbial colonization that includes amoebae, but this will be controlled by low temperatures. Significant concentrations will develop only in situations where temperatures rise above 20 °C for prolonged periods.

Growth and/or recontamination in distribution systems

At temperatures between 20 °C and 50 °C, legionellae frequently colonize water distribution systems. The main sites of colonization, bacterial growth, and

contamination are the pipework in buildings, boilers (especially if they contain sediment), membrane expansion vessels and reservoirs inside buildings, as well as the fittings, outlets, and accessory devices connected to water-supply systems. Special mention should be made of medical and dental equipment containing or supplied with water, because it is likely to be used on, or in the vicinity of, susceptible individuals. Colonization is enhanced at temperatures above 25 °C, by stagnation, and by formation of biofilms that include protozoa and have an elevated iron content. All these features are common in the warm-water distribution systems of large buildings, including hospitals and other clinical establishments.

Measurable inactivation of legionellae begins at a temperature of 50 °C: for *L. pneumophila*, decimal reduction times of 80–111 minutes at 50 °C, 27 minutes at 54 °C, 19 minutes at 55 °C, 6 minutes at 57.5 °C, and 2 minutes at 60 °C have been recorded (Dennis, Green & Jones, 1984; Schulze-Röbbecke, Rödder & Exner, 1987).

Prolonged stagnation (of several months, for instance during building construction or over holiday periods) resulting in a heavy microbial load has been reported on several occasions when water has been identified as the source of infection (Dondero et al., 1980; Kramer et al., 1992; Breiman, 1993; Mermel et al., 1995; Straus et al., 1996). Pressure-compensation vessels (shock absorbers) also provide the conditions for stagnation (Memish et al., 1992) and should be positioned on the cold-water (i.e. intake) side of hot-water installations.

The concentration of assimilable organic carbon (AOC) in water seems to have less influence on the growth of *Legionella* than on the formation of biofilms. Legionellae are not observed in the absence of other microorganisms. Biofilm formation is encouraged not only by elevated AOC levels but also by certain materials present in a plumbing system. Since legionellae are iron-dependent, it is to be expected that the use of iron piping would encourage their growth; *Legionella*-contaminated water frequently contains high levels of iron as the result of corrosion. However, in a study on the prevalence of legionellae in private homes, the organisms were found only in houses with copper pipework (Tiefenbrunner et al., 1993), and many hospitals in the United Kingdom that have experienced outbreaks of Legionnaires diseases also had copper plumbing. It therefore seems that avoidance of iron or steel pipework does not protect against colonization by *Legionella*.

Materials that promote biofilm formation by nutrients that migrate to surfaces in contact with the water should not be used in water installations, whether as coatings, fillings, or sealants for pipes, reservoirs, or containers, or for devices such as membranes of pressure-compensation vessels, tap washers, etc. (Colbourne et al., 1984; Niedevelde, Pet & Meenhorst, 1986). Biofilms will also form, however, on inert surfaces, albeit more slowly and less extensively, so that the material of which the surface is composed is actually less important than the size of the biofilm-bearing surface. The larger the surface that is available for bacterial growth in a water system, the more likely it is to become colonized by legionellae;

thus small water systems in single dwellings are much less likely to become colonized than large systems in, for example, hotels or hospitals.

Control of legionellae in potable-water systems

Prevention

Entry into a potable-water system of single bacteria or bacteria-carrying amoebae from the public supply system, or during construction or repair, must always be considered as a possibility. Prevention of significant bacterial growth is best achieved by keeping water cool (preferably below 15 °C) and flowing, or hot (at least 55 °C) and flowing. Mains drinking-water supplies can be kept free of significant levels of *Legionella* by chlorination: a concentration of 0.2 mg/litre free chlorine will keep levels below 1 cfu/100 ml, indicating that no active replication is occurring. The low levels of legionellae occasionally found in public water supplies have never been shown to constitute a health risk. Inside buildings, however, the residual chlorine (if any) carried over from the public supply will not prevent growth of these organisms, and additional measures are required to prevent water stagnation at temperatures that will allow bacterial growth.

The cold water supply should be kept cool, with temperatures at outlets not exceeding 20 °C. Pipework, storage tanks, and devices such as water softeners should be insulated against heat gain and should never be situated in rooms where the temperature is constantly high. Hot water must be stored and distributed at a temperature of at least 50 °C throughout the system. It is recommended that the water is heated to, and stored at, 60 °C, and that it attains 50 °C (NHMRC, 1996; Health and Safety Executive, 2000), 55 °C (DVGW, 1996), or 60 °C (Gezondheidsrad, 1986) at taps after running for no more than 1 minute; temperatures inside boilers and recirculation systems should be similar. For this purpose, the design and construction of the hot-water system must meet certain requirements that are otherwise unnecessary. Water temperatures inside calorifiers and tanks must reach 60 °C throughout, including at the bottom, at least once a day. The calorifier must be able to achieve this temperature consistently, even during periods of high demand. For the purpose of thermal disinfection, the calorifier must produce sufficient amounts of water to flush all outlets in the building with water at 70 °C. Tanks must be accessible for cleaning and the accumulation of sludge must be avoided. Connected tanks, filters, and other appliances must be scrutinized for their potential to promote *Legionella* growth. Pipework should be as short and easy to survey as possible and should avoid “dead ends” and other zones of stagnation. Outlets should be fitted with mixer taps to reduce the risk of scalding.

Point-of-use water heaters have been proposed as a means of obviating the need for hot-water storage and distribution systems (Muraca, Yu & Goetz, 1990). Even these, however, are not totally failsafe, since growth of legionellae can occur at the outlets (Sellick & Mylotte, 1993).

Since stagnation will give rise to elevated colony counts (and also frequently to high concentrations of legionellae in the water within pipes and reservoirs), it has been proposed that water-supply systems should be drained when there is to be an extended period (e.g. weeks) during which there will be no water consumption.

Particular care should be taken to protect plumbing systems during the construction of new hospital buildings. Before a new or renovated hospital or similar building is opened, the water in the supply system should be tested for microbiological quality, including the presence of *Legionella*.

Eradication/disinfection

When hot-water systems develop problems that cannot be identified or repaired, it is often difficult to keep them permanently free of elevated concentrations of legionellae. Continuous or intermittent treatment for purposes of disinfection or permanent eradication may then be advisable. Techniques for the eradication of *Legionella* include the following:

- thermal disinfection: heating and flushing
- UV irradiation
- use of chlorine, chlorine dioxide, chloramine, ozone, or iodine
- metal ionization (copper and silver).

Raising the water temperature to at least 60°C (Health and Safety Executive, 2000) is the most reliable means of eradicating legionellae from a water-supply system, although the exact temperature and the length of time necessary for heating and flushing the system, including the outlets, remain matters of some debate (Dennis, Green & Jones, 1984; Snyder et al., 1990). An 8 log₁₀ reduction in *L. pneumophila* has been demonstrated within 25 minutes at 60°C, 10 minutes at 70°C, and 5 minutes at 80°C. The most resistant species—*L. micdadei*—is about twice as resistant to thermal disinfection as *L. pneumophila* (Stout, Best & Yu, 1986). This is consistent with the finding that *Legionella* in a hospital water system could not be eradicated by raising the temperature in the hot-water tank to 60°C; however, raising the temperature in the tank to 77°C (which produced 50–60°C in the system as a whole) successfully eradicated the organism (Best et al., 1983). At 50–60°C in a model plumbing system, a 7 log₁₀ reduction in legionellae occurred in under 3 hours (Muraca, Stout & Yu, 1987).

From the observation of Stout, Best & Yu (1986), it follows that thermal disinfection of plumbing systems requires the water in boilers and tanks to be heated to 70°C and taps (outlets) to be kept open for 30 minutes; see also Plouffe et al. (1983). This measure should succeed in eliminating *Legionella* for some weeks. Reappearance of the organisms in the water is usually accompanied or preceded by elevated concentrations of other bacteria, resulting in an elevated heterotrophic plate count (HPC), i.e. >100 colony-forming units/ml (cfu/ml), which is more easily monitored than the concentration of legionellae. Chlorination of the water, however, may make this indicator useless, because chlorine

does not affect legionellae to the same extent as HPC (Zacheus & Martikainen, 1996).

Compared with other Gram-negative bacteria, the legionellae are highly susceptible to UV irradiation (Antopol & Ellner, 1979). In the dark, a 90% reduction has been achieved at 5 W·s/m², 99% at 10 W·s/m², and 99.9% at 16 W·s/m². On exposure to photoreactivating light, however, doses at least 3 times higher are needed because of the organism's effective light-dependent DNA repair system (Knudson, 1985). In hospitals, UV irradiation units installed near "points of use", together with prefiltration systems to prevent accumulation of scale, have been successful in keeping water outlets free of legionellae (Farr et al., 1988; Liu et al., 1995). However, these units are effective only over short distances.

Chlorine is much better tolerated by *Legionella* spp. than by many other bacteria, including *Escherichia coli*. Achieving a given reduction in different species of media-grown *Legionella* required more than 40 times longer than the same reduction in *E. coli* (Kuchta et al., 1983). Tap-water-adapted strains have been reported to be 68 times as resistant as *E. coli* when computed as a product of concentration and time (CT), and experiments using iodine suggest that cultures associated with stainless-steel surfaces are even more resistant (Cargill & Pyle, 1992). The resistance of *Legionella* to chlorine is further enhanced by inclusion of the organisms in amoebae or by growth in biofilms (Kuchta et al., 1993), and it is therefore unsurprising that legionellae have repeatedly been found in chlorinated water that complies with microbiological standards for drinking-water. In reality, the calculation of CT values in laboratory experiments with cultured legionellae is an inadequate indication of resistance to chlorine and other antimicrobial agents.

A chlorine concentration of 2 mg/litre will kill free legionellae (Kuchta et al., 1993) and appears to be sufficient to keep the organisms at low levels in hot water (Snyder et al., 1990; Grosserode et al., 1993); even at chlorine levels of 4 mg/litre, however, amoebae containing *L. pneumophila* will liberate viable organisms (Kuchta et al., 1993). Continuous hyperchlorination (>2 mg/litre) may cause corrosion of pipes and formation of trihalomethanes as by-products (Helms et al., 1988; Grosserode et al., 1993). Nevertheless, supplementary chlorination of the hot-water supply may produce satisfactory results in many situations.

There have been fewer studies of chlorine compounds than of chlorine itself. Chlorine dioxide is probably more effective than chlorine because of its superior oxidative power and effect on biofilms (Walker et al., 1995; Hamilton, Seal & Hay, 1996). Chloramines have a slower action than chlorine but greater stability; Cunliffe (1990) reported that legionellae were much more sensitive than *E. coli* to monochloramine—a compound that is used in Australia to control the growth of *Naegleria fowleri*. In a comparison of hospitals that had reported Legionnaires disease with others that had not, Kool, Carpenter & Fields (1999) revealed that outbreaks were 10 times as likely in a hospital with residual free chlorine in its water than in one where the residual disinfectant was chloramine.

Electrolytically generated copper and silver ions have been shown to be effective in reducing legionellae *in vitro* (Landeem, Yahya & Gerba, 1989). At 45 °C, a 5 log₁₀ reduction in legionellae was achieved after 1 hour with silver and copper ion concentrations of 80 and 800 µg/litre respectively, and after 24 hours with concentrations of 20 and 200 µg/litre (Rohr, Senger & Selenka, 1996). Lin et al. (1996) have also demonstrated a synergistic effect of copper and silver ions. Copper/silver ionization has been used successfully in hot-water recirculating systems, reducing *Legionella* concentrations to 10–100 cfu/litre (Colville et al., 1993; Liu et al., 1994; Selenka et al., 1995; Rohr et al., 1996), although there is still no proof of continuing efficiency with prolonged use.

The resistance of legionellae to ozone is comparable to that of *E. coli* and *Pseudomonas aeruginosa* (Domingue et al., 1988): at an ozone concentration of about 0.3 mg/litre, a 4–5 log₁₀ reduction in the number of organisms was achieved within 20 minutes (Edelstein et al., 1982; Domingue et al., 1988). However, results for the use of ozone to eradicate legionellae from water systems remain ambiguous. It is probably difficult to achieve a sufficient contact time, since adequate levels of residual ozone will not persist in extended domestic water-supply systems. Additional considerations include the safety and corrosive effects of ozone, and compliance with local regulations.

Although each of the disinfection techniques described in this section has proved effective in reducing *Legionella* under controlled conditions, there are differences in their costs and in their suitability for use in large domestic water-distribution systems. In the event of a disease outbreak or other situation requiring immediate action, heat flushing—alone or combined with hyperchlorination—may be the most appropriate measure to apply. To prevent recurrence, the affected system should be checked for any peculiarities of design or operation that predispose to *Legionella* colonization, and these should be corrected, if possible, before any further action is taken.

Conclusions and recommendations

Health risk assessment

The risk of infection following exposure to *Legionella* is difficult to assess and remains a matter of some debate (O'Brien & Bhopal, 1993). Since *Legionella* is ubiquitous in both natural and man-made environments, it must be supposed that most people are frequently exposed, at least to single organisms. Generally, there is either no reaction to such exposure or asymptomatic production of antibodies. Drinking-water from natural sources and from public supplies may carry single organisms or *Legionella*-containing amoebae but, outside hospitals, there are no reports of outbreaks or recurrent cases of disease following consumption or use of drinking-water that has been kept cool and not subjected to prolonged periods of stagnation. However, the inference to be drawn from the many reported outbreaks and documented single cases is that

inhalation of small numbers of bacteria, or aspiration following ingestion, will lead to disease.

Risk of infection is acknowledged to be high among transplant patients, patients receiving high-dose steroid treatment or intensive care, individuals being fed by nasogastric tube, and people with malignancies and end-stage renal disease. Special measures of protection and surveillance are essential for people in these categories. Increased susceptibility during outbreaks has also been observed among males, diabetic patients, the elderly, and people with reduced resistance to respiratory disease (e.g. smokers). Nonetheless, no unequivocal dividing line between those at risk and those not at risk has yet been established.

Risk management strategies

Most outbreaks reported to date have been associated with cooling towers, evaporative condensers of air-conditioning devices, potable water at elevated temperatures (especially in hospitals and hotels), hot whirlpool and spa baths, nebulizers, and certain potting composts. The greatest risk seems to be associated with water subjected to prolonged periods of stagnation and in systems that are frequently maintained at temperatures of 25–50 °C; this range of temperatures should therefore be avoided as far as possible.

Water systems—particularly cooling towers and evaporative condensers—should be designed, constructed, and operated in such a way that microbial growth is minimized. High water temperature is the most efficient approach to both intermittent disinfection and continuous control. In hot-water distribution systems, water temperatures should exceed 60 °C in boilers, reservoirs, and circulating pipes, and reach 50 °C at outlets. Continuous surveillance and disinfection have been proposed for water systems in hospitals and in public swimming pools, hot whirlpool and spa baths and the like, and for medical and dental equipment that uses water. However, opinion continues to be divided on this issue (Centers for Disease Control and Prevention, 1997), and there is no generally accepted threshold limit for the concentration of legionellae in water. Surveillance of the drinking-water and hot-water supply systems in hospitals is recommended by some (Allegheny County Health Department, 1997), and considered prudent in institutions for the elderly and, possibly, in large hotels.

Total prevention of sporadic infections is impossible, because of the widespread occurrence of *Legionella* in all environments. In hospitals, however, all clinical specimens from patients with symptoms of pneumonia should be examined for *Legionella*, *Legionella* antibodies, and *Legionella* antigen. Transplant patients should be scrupulously protected from exposure to *Legionella* during immunosuppression; their drinking-water should be sterilized, and sterilized water should be used for washing these patients.

There are insufficient data to support widespread disinfection of water-supply systems in the absence of any linkage to *Legionella* infections, but in all cases of nosocomial pneumonia every effort must be made to identify the source

of infection and implement measures to interrupt transmission. Continuous monitoring of the water, however, is advocated only when antimicrobial measures have to be checked.

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Protozoan parasites (*Cryptosporidium*, *Giardia*, *Cyclospora*)¹

Description

Several species of parasitic protozoa are transmitted through water, with *Giardia intestinalis* and *Entamoeba histolytica/dispar* being among the most important intestinal parasites worldwide. Morbidity, and particularly mortality, rates for *E. histolytica/dispar* are high, especially in nonindustrialized countries. More information on *Entamoeba* can be found in Volume 2 of the WHO *Guidelines for drinking-water quality*. A wide variety of free-living amoebae occur in water, but only *Naegleria fowleri* and *Acanthamoeba* spp. have been identified as pathogenic for man. *N. fowleri* may be present in thermally polluted waters and sporadically causes fatal primary amoebic meningoencephalitis; however, only one outbreak has been related to a drinking-water supply system (Marshall et al., 1997). *Acanthamoeba* spp. can be found throughout the aquatic environment; these organisms cause sporadic cases of keratitis in wearers of contact lenses after exposure to contaminated recreational water and contact lens cleaning fluids (Marshall et al., 1997). Drinking-water taps were identified as the source of contamination when home-made lens-cleaning solutions were found to contain *Acanthamoeba*

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(Seal, Hay & Kirkness, 1995). *Acanthamoeba* has also been suggested as a vehicle for environmental transmission of *Legionella* bacteria (Campbell et al., 1995).

The growth in the number of severely immunocompromised individuals— as a result of the AIDS epidemic, cancer chemotherapy, and organ transplants— has been paralleled both by the increasing prevalence of opportunistic infections and by greater recognition of the disease-causing potential of various other intestinal protozoan parasites, such as *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and microsporidia, as human pathogens. The first human cases of cryptosporidiosis were reported in 1976 (Meisel et al., 1976; Nime et al., 1976). *Cryptosporidium* was initially thought to be an opportunistic pathogen of immunocompromised persons, but a number of waterborne outbreaks, plus frequent cases in immunocompetent individuals, have disproved this. Indeed, *C. parvum* is now one of the most commonly identified intestinal pathogens throughout the world. Its occurrence is dependent on factors that include season, and the age and other demographic characteristics of a population: among children aged 1–5 years with diarrhoea, *C. parvum* may be the most frequently found pathogen (Palmer, 1990).

Cyclospora cayetanensis—originally referred to as “cyanobacterium-like bodies”—has recently been recognized as a waterborne pathogen and reclassified (Bendall et al., 1993; Ortega et al., 1993). It has been associated with several waterborne outbreaks worldwide.

There are almost 1000 species of microsporidia, widely distributed in nature (Stewart & Osborn, 1996). Microsporidia have long been recognized as pathogens in fish, birds, and some mammals, but several species have recently been identified as the cause of disease in severely immunocompromised humans. These organisms are associated primarily with infections of the intestinal tract, but dissemination to the biliary, urinary, and respiratory tracts may occur and some species have been implicated in ocular infections in immunocompetent persons. The mode of transmission is still unclear, but a faecal–oral route is likely. The persistence of these organisms in water, their resistance to disinfection, and their small size (some as small as 1–2 μm) suggest that waterborne transmission must be considered possible, especially for immunocompromised individuals, although this has not yet been demonstrated.

Toxoplasma gondii is an intracellular coccidian parasite that has long been recognized as a human pathogen. Felines are the definitive host and are infected primarily by the consumption of infected mammals and birds, which act as secondary hosts. In secondary hosts, the parasite becomes encysted in muscle and brain tissue; only felines carry the parasite in the intestinal tract and shed oocysts that sporulate in the environment. The oocysts are 10–12 μm in diameter and can survive in water and moist soils for long periods of time. Consumption of undercooked meats and raw milk and contact with feline faeces (in cat litter or sand boxes) are the primary sources of *Toxoplasma* infections in humans (Stewart & Osborn, 1996). One waterborne outbreak has been reported, and was believed to have resulted from contamination of water by cat faeces (Bowie et al., 1997).

Since *Cryptosporidium parvum*, *Giardia intestinalis*, and *Cyclospora cayotensis* are the parasites of primary concern in the area of drinking-water supply, and much information on waterborne transmission is available from recent research, it is on these three organisms that the remainder of this section concentrates.

Significance of *Cryptosporidium* and *Giardia* as waterborne pathogens

Oocysts of *Cryptosporidium* and cysts of *Giardia* occur in the aquatic environment throughout the world. They have been found in most surface waters, where their concentration is related to the level of faecal pollution or human use of the water (Hansen & Ongerth, 1991; LeChevallier, Norton & Lee, 1991). The environmentally robust (oo)cysts are very persistent in water (DeReignier et al., 1989; Robertson, Campbell & Smith, 1992; Chauret et al., 1995) and extremely resistant to the disinfectants commonly used in drinking-water treatment (Hibler et al., 1987; Korich et al., 1990; Finch et al., 1993a, 1993b). These characteristics, coupled with the low numbers of (oo)cysts required for an infection (Rendtorff, 1954; Dupont et al., 1995; Okhuysen et al., 1998), place these organisms among the most critical pathogens in the production of safe drinking-water from surface water. Well protected groundwaters that are not mixed with surface water or otherwise contaminated are free of these and other enteropathogens. If abstraction, treatment, and distribution systems for these waters are properly designed and operated, the risk of faecal contamination is very low and there will be no waterborne transmission of parasitic protozoa. Groundwaters that mix with surface water or other sources of contamination (e.g. surface run-off) may contain low levels of *Cryptosporidium* and *Giardia* (Hancock, Rose & Callahan, 1997) and give rise to waterborne illness (Craun et al., 1998). Filtration of such waters is essential to the production of safe drinking-water: treatment by disinfection alone offers no protection against *Cryptosporidium* and only limited protection against *Giardia*.

Many waterborne outbreaks of giardiasis and cryptosporidiosis have been reported in industrialized countries (Craun, 1990; MacKenzie et al., 1994; Craun et al., 1998). In these outbreaks, (oo)cysts have entered the drinking-water because of surface-water treatment failure, contamination of the source water, and leakage into the distribution system. In a significant number of these outbreaks, the drinking-water implicated as the cause complied with the WHO Guidelines for *Escherichia coli* levels and turbidity (Craun 1990; Craun et al., 1998), but deviations from normal raw water quality or treatment operations recommended in the *Guidelines* were identified. However, in an outbreak in Las Vegas, USA, that was traced to drinking-water, no abnormalities in treatment operations or in the quality of raw or treated water were detected (Goldstein et al., 1996).

The fact that outbreaks occur in the absence of any warning signal from the routine water quality monitoring for coliforms points to a severe limitation of

coliform level as an indicator for microbiological safety of drinking-water. Additional means of safeguarding drinking-water are therefore imperative.

Cryptosporidium parvum

Taxonomy

Members of the genus *Cryptosporidium* (Apicomplexa, Cryptosporidiidae) are small coccidian protozoan parasites that infect the microvillous region of epithelial cells in the digestive and respiratory tracts of vertebrates. Several species of *Cryptosporidium* have been described and appear to be specific for a class of vertebrates: *C. parvum*, *C. muris*, *C. felis*, and *C. wrairi* infect mammals, *C. baileyi* and *C. meleagridis* infect birds, *C. serpentis* infects reptiles, and *C. nesorum* infects tropical fish. Infections in humans are almost exclusively caused by *C. parvum*, although this species is also frequently found in infections of cattle and sheep and causes infections in many other mammal species.

Life cycle

Oocysts, the environmentally resistant transmission stage of the parasite, are shed by infected hosts with their faeces (Fayer & Ungar, 1986; Fayer, Speer & Dubey, 1997) and are immediately infectious. They may remain in the environment for very long periods without loss of infectivity: a very robust oocyst wall protects the sporozoites inside against physical and chemical damage. When an oocyst is ingested by a new host, excystation—opening of the suture in the oocyst wall—is triggered by the body temperature and the interaction with stomach acid and bile salts. Four motile sporozoites are released, which infect the epithelial cells of the small intestine, mainly in the jejunum and ileum. The parasite infects the apex of the epithelial cells, residing beneath the cell membrane but outside the cytoplasm. The sporozoites undergo several transformations in an asexual (merogony) and a sexual (gametogony) reproduction cycle; it is the latter that generates the oocysts.

Oocysts of *C. parvum* are spherical, with a diameter of 4–6 µm, and may be either thick- or thin-walled oocysts. Thin-walled oocysts may excyst within the same host and start a new life cycle (autoinfection). This can lead to heavily infected intestinal epithelia and result in malabsorptive or secretory diarrhoea. Thick-walled oocysts are excreted with the faeces.

Pathogenicity

Infection studies in healthy human volunteers demonstrated a clear relationship between probability of infection and the ingested oocyst dose of a bovine *C. parvum* strain (Dupont et al., 1995). At the lowest dose (30 oocysts), the probability of infection was 20%; at a dose of 1000 oocysts, probability increased

to 100%. When the dose–response data are fitted with an exponential model, the probability of infection (P_1) is described by:

$$P_1 = 1 - e^{-r \times \text{dose}}$$

where r , the dose–response parameter, is 0.004005 (95% CI 0.00205–0.00723) for this *C. parvum* strain (Teunis et al., 1996).

This approach assumes that ingestion of even a single oocyst results in a distinct probability (0.5%) of infection. Although there was a clear dose–response relation for infection, occurrence of symptoms of intestinal illness in the volunteers was not dose-related.

The disease

The average incubation period varies widely but is usually about 7 days (Ungar, 1990; Dupont et al., 1995). Watery diarrhoea is the most prominent symptom of intestinal *C. parvum* infection (Fayer & Ungar, 1986; Ungar, 1990), and the frequent and copious bowel movements can cause dehydration and weight loss (Arrowood, 1997). Other symptoms are nausea, abdominal cramps, vomiting, and mild fever. During the 1993 Milwaukee waterborne outbreak, which involved 400 000 patients, MacKenzie et al. (1994) compared clinical data from cases detected by (passive) laboratory surveillance with cases detected by (active) telephone surveys. Patients who submitted a stool sample for laboratory diagnosis suffered more serious disease, as manifested by the higher frequency of fatigue, loss of appetite, nausea, fever, chills and sweats, and vomiting.

In immunocompetent individuals, the infection is limited by the immune response that eventually clears the parasite. Infections in patients with defective cellular immune response (congenital or due to AIDS or chemotherapy) or humoral immune response (in congenital hypogammaglobulinaemia) are persistent and heavy, suggesting that both types of immune response are needed to limit and clear the infection. Several animal studies suggest that the immune response protects against reinfection (Zu et al., 1992), and protective immunity in humans is indicated by the large numbers of asymptomatic carriers in countries with a high prevalence of cryptosporidiosis. Moreover, infected volunteers who were challenged with the same strain one year after initial infection were significantly less susceptible to reinfection (Okhuysen et al., 1998); occurrence of diarrhoea was similar in both exposures, but the illness was less severe in the reinfected volunteers, which indicates some degree of protective immunity.

The duration of the infection is generally 7–14 days in immunocompetent individuals, but a median duration of 23–32 days has also been reported (van Asperen et al., 1996). The peak intensity of oocyst shedding, with an average concentration of 10^6 /g, coincides with the peak intensity of clinical symptoms. Oocyst shedding lasts for at least 2 weeks in 82% of infected people, 3 weeks in 42%, and 4 weeks in 21% (Baxby, Hart & Blundell, 1985). Again, there is a difference between cases under laboratory surveillance (duration 2–4 weeks) and

cases in the general population (duration typically 3–6 days). Relapses of diarrhoea are common: up to five additional episodes in 40–70% of patients have been reported in both population-based (outbreak) studies and in studies in volunteers. This phenomenon considerably increases both the mean duration of disease and its variability.

Mortality in immunocompetent patients is generally low. In immunodeficient individuals, however, the infection can be persistent and severe (Ungar, 1990), resulting in very profuse diarrhoea and consequent severe dehydration. Severe infections have been reported in patients with concurrent infections (principally AIDS, but also measles and chickenpox), people with congenital immune deficiencies, patients receiving immunosuppressive drugs (for cancer therapy, transplants, or skin lesions), and malnourished individuals (Fayer, Speer & Dubey, 1997). It is also reported that pregnancy may predispose to *Cryptosporidium* infection (Ungar, 1990).

The prevalence of cryptosporidiosis in AIDS patients in industrialized countries is around 10–20% (Current & Garcia, 1991). In the absence of an effective immune response, the infection may spread throughout the entire intestinal tract and to other parts of the body (gall bladder, pancreas, respiratory tract). No consistently effective therapeutic agent has been found (Blagburn & Soave, 1997). Immunotherapy with monoclonal antibodies or hyperimmune bovine colostrum has been reported to resolve diarrhoea, at least temporarily, in AIDS patients (Riggs, 1997), and similar findings have been reported for other chemotherapeutic agents (azithromycin, paromomycin) (Blagburn & Soave, 1997).

The severe dehydration, the spread of infection, and the lack of an effective therapy lead to high mortality in immunodeficient patients, although this has not been accurately quantified. In one study in the UK, 19% of AIDS patients with cryptosporidiosis were thought to have died from the infection (Connolly et al., 1988). Another study compiled case reports of cryptosporidiosis and found a mortality rate of 46% in AIDS patients and 29% in patients with other immunodeficiencies (Fayer & Ungar, 1986).

Prevalence

In stool surveys of patients with gastroenteritis, the reported prevalence of *Cryptosporidium* is 1–4% in Europe and north America and 3–20% in Africa, Asia, Australia, and south and central America (Current & Garcia, 1991). Peaks in the prevalence in developed countries are observed in spring (Casemore, 1990) and in the late summer (van Asperen et al., 1996).

Numbers of asymptomatic carriers, as determined by stool surveys, are generally very low (<1%) in industrialized countries (Current & Garcia, 1991), although higher rates have been reported in day-care centres (Lacroix et al., 1987; Crawford & Vermund, 1988; Garcia-Rodriguez et al., 1989). Routine bile endoscopy suggests a higher prevalence of asymptomatic carriage: 13% of

non-diarrhoeic patients were shown to carry *Cryptosporidium* oocysts (Roberts et al., 1989). High rates of asymptomatic carriage (10–30%) are common in non-industrialized countries (Current & Garcia, 1991). Seroprevalence rates are generally higher than faecal carriage rates, from 25–35% in industrialized countries to 95% in south America (Casemore, Wright & Coop, 1997), increase with age (Zu et al., 1992; Kuhls et al., 1994), and are relatively high in dairy farmers (Lengerich et al., 1993) and day-care centre attendants (Kuhls et al., 1994).

Routes of transmission

A major route of infection with *Cryptosporidium* is person-to-person transmission, as illustrated by outbreaks in day-care centres (Fayer & Ungar, 1986; Casemore, 1990; Cordell & Addiss, 1994) and the spread of infection within the households of children attending these centres. Sexual practices involving oro-anal contact also involve a high risk of exposure to the organism. *Cryptosporidium* can also be transmitted from mammals—especially newborn animals—to humans, and many infections have been derived from contact with infected calves and lambs (Casemore, 1990). Domestic pets can be infected with oocysts, but do not appear to be important sources of human infection (Casemore, Wright & Coop, 1997; Glaser et al., 1998). Indirect person-to-person or zoonotic transmission may occur through contaminated water used for recreation (e.g. swimming pools) or through food and drinks (raw meat and milk, farm-made apple cider) (Casemore, Wright & Coop, 1997).

Waterborne outbreaks of cryptosporidiosis have been attributed to contaminated drinking-water, from both surface-water and groundwater sources (Craun, 1990; Mackenzie et al., 1994; de Jong & Andersson, 1997), and to recreational water, including swimming pools (Joce et al., 1991; MacKenzie, Kazmierczak & Davis, 1995; van Asperen et al., 1996; Anon., 1998; Kramer et al., 1998).

Outbreaks caused by drinking-water have been attributed to contamination of the source water by heavy rainfall or snow-melt (Richardson et al., 1991; Pett, Smith & Stendahl, 1993; MacKenzie et al., 1994), to sewage contamination of wells (d'Antonio et al., 1985; Kramer et al., 1996), to inadequate treatment (Richardson et al., 1991; Craun et al., 1998) or treatment deficiencies (Badenoch, 1990; Leland et al., 1993; Craun et al., 1998), and to combinations of these factors (MacKenzie et al., 1994). Leakages and cross-connections in water-distribution systems have also caused outbreaks of cryptosporidiosis (Craun, 1990; de Jong & Andersson, 1997; Craun et al., 1998). As many as 400 000 people have been affected by a cryptosporidiosis outbreak transmitted through drinking-water.

A wide range of oocyst concentrations in drinking-water have been detected during outbreaks of disease (Haas & Rose, 1995). However, tests carried out during an outbreak are usually too late to determine the concentration that triggered the outbreak. To obtain “historical” data on the occurrence of oocysts in drinking-water, researchers have attempted to detect oocysts in ice (MacKenzie et al., 1994), in in-line filters (van Asperen et al., 1996), and in sediments of

water-storage tanks (Pozio et. al., 1997). The results probably represent underestimates of the concentrations that caused the outbreaks. For the 1993 Milwaukee outbreak, however, Haas & Rose (1994) showed (with certain assumptions) that the measured concentration in drinking-water was close to the value predicted on the basis of the attack rate, water consumption, and dose–response relationship.

Low oocyst concentrations in drinking-water have also been found in situations where there was no evidence for the occurrence of an outbreak (LeChevallier, Norton & Lee, 1991; Karanis & Seitz, 1996; Rose, Lisle & LeChevallier, 1997; McClellan, 1998). Current detection methods do not allow the determination of pathogenicity of oocysts in water. Detection of oocysts in treated water should therefore, wherever possible, prompt further tests to confirm the presence of (viable) *C. parvum* oocysts. Whether or not the capacity for such tests exists, every effort should be made to thoroughly examine other water quality parameters that may point to faecal contamination and to trace both the source of contamination and the operational conditions that resulted in the presence of oocysts in the drinking-water. If the presence of *C. parvum* oocysts is confirmed, control measures (improved source protection and/or water treatment) should be instituted; ideally, an epidemiological study should also be undertaken to determine whether there has been significant waterborne transmission. Lack of capacity to confirm the pathogenicity or viability of oocysts should not delay or prevent further investigation or remedial action if a significant threat to public health is suspected.

Giardia intestinalis

Taxonomy

Giardia is a flagellated protozoan the taxonomy and host specificity of which remain the subject of considerable debate. The organism has been found in more than 40 animal species (Meyer, 1994). Nowadays, five species of *Giardia* are established in the scientific literature, including the three species proposed by Filice (1952)—*G. muris* in rodents, birds, and reptiles, *G. intestinalis* (syn: *duodenalis*, syn: *lamblia*) in mammals (including man), rodents, reptiles, and possibly birds, *G. agilis* in amphibians—*G. ardae* in the great blue heron (Erlandsen et al., 1990), and *G. psittaci* in the budgerigar (Erlandsen & Bemrick, 1987). A morphologically distinct *Giardia* isolated from the straw-necked ibis (Forshaw, 1992) was later suggested to be a strain of *G. ardae* (McRoberts et al., 1996).

Giardia is thought to be predominantly asexual, and the species concept—defined on the basis of sexually reproductive compatibility—is therefore difficult to apply. The high degree of genetic heterogeneity found in human and animal isolates (Nash et al., 1985; Andrews et al., 1989; Meloni, Lymbery & Thompson, 1989; Morgan et al., 1994) makes speciation uncertain and suggests that

Giardia is a clonal parasite (Tibayrenic, 1994). *G. intestinalis* can be subdivided by several techniques into two groups (Homan et al., 1992, 1994). It is still uncertain how, or even whether, this heterogeneity is related to host specificity and pathogenicity of *Giardia*.

Life cycle

The life cycle of *Giardia* is simple (Feely, Holberton & Erlandsen, 1990; Meyer, 1994). As with *Cryptosporidium*, the parasite is shed with the faeces as an environmentally robust cyst, which can then be transmitted to a new host. In the duodenum of the new host, the trophozoite emerges from the cyst and undergoes a mitotic division. Each of the two trophozoites produced in this way attaches to the epithelial cells by means of an adhesive disc, then feeds on the epithelial cells. The trophozoites detach from the epithelial cells, probably because of the rapid turnover (72 hours) of these cells, and undergo mitotic division in the intestinal lumen. During periods of diarrhoea, these trophozoites may be transported with the intestinal contents and excreted, but do not survive long outside the host. Some of the trophozoites encyst during the passage through the intestine and leave the host with the faeces as cysts. In formed stools, cysts are encountered more often than trophozoites.

Giardia intestinalis cysts are elliptical, 8–12 µm long and 7–10 µm wide. The cyst wall is 0.3–0.5 µm thick and has a fibrillous structure. Two to four nuclei are found in each cyst, together with axonemes of the flagella of the trophozoite.

Pathogenicity

Studies in human volunteers revealed a dose–response relationship between the probability of infection and the ingested dose of *G. intestinalis* cysts (Rendtorff, 1954), although no data on the viability of the ingested cysts were provided. A dose of 10 cysts resulted in an infection in 100% (2/2) of the volunteers.

The probability of infection, P_i , has been described with an exponential model (Rose et al., 1991b):

$$P_i = 1 - e^{-r \times \text{dose}}$$

where r , the dose–response parameter, is 0.0199 (95% CI: 0.0044–0.0566). Although 53% of the volunteers became infected in this study, and changes in bowel motions were observed, none of the volunteers developed symptoms of giardiasis. The infection-to-illness ratio varies between isolates, as shown by the different response of volunteer subjects to two different isolates from symptomatic human infections in a study by Nash et al. (1987). Other factors, such as age, nutritional status, predisposing illness, and previous exposure, determine the outcome of an infection (Flannagan, 1992). Asymptomatic carriage appears to be the most common form of infection with *Giardia* (Farthing, 1994); 16–86% of infected individuals are asymptomatic.

The mechanism by which *Giardia* causes diarrhoea and malabsorption is still unclear. The organism could act as a physical barrier, but the area covered by trophozoites is probably too small to affect the absorption of nutrients. There is no evidence for the production of toxins (Buret, 1994). *Giardia* infections appear to affect the activity of gut enzymes (lactase, disaccharidase), damage the mucosal surface (causing shortening of crypts and villi), and give rise to overgrowth of bacteria (Tomkins et al., 1978) or yeasts (Naik et al., 1978) in the small intestine.

The disease

The time between infection and the appearance of *Giardia* cysts in the stool is 12–19 days (Jokipii, Hemila & Jokipii, 1985). Symptoms appear between 1 and 75 days after infection, but generally at 6–15 days, coinciding with the appearance of *Giardia* in stool (Rendtorff, 1954; Brodsky, Spencer & Schultz, 1974). The most prominent symptoms are diarrhoea (fatty, yellowish) weakness, weight loss, abdominal pain, and—to a lesser extent—nausea, vomiting, flatulence, and fever. In most cases, the infection is acute and self-limiting, with a duration of 2–4 weeks. However, a significant proportion of the infected population (estimated at 30–50%) will then develop chronic infection with intermittent diarrhoea (Farthing, 1994); weight loss can be substantial (10–20%) in this group. The ability of *Giardia* to change the surface epitopes of the trophozoites during infection (Nash, 1992) may play a role in the occurrence of chronic infections. There is evidence that infection of children with *Giardia* causes failure to thrive by impairing the uptake of nutrients (especially fats and vitamins A and B₁₂) (Farthing, 1994; Hall, 1994).

Excretion of cysts varies between 10^6 and 10^8 per gram of stool, as determined in positive stool samples (Tsuchiya, 1931), but a significant proportion of the stool samples do not contain detectable levels of *Giardia*. Excretion patterns vary with the host and with the isolate.

Prevalence

Giardia infections are very common in children in developing countries (Farthing, 1994; Rabbani & Islam, 1994). In developed countries, prevalence peaks at the age of 1–4 years (Flanagan, 1992) and again in the 20–40-year age group, partly through caring for young children and partly as a result of travelling.

In developing countries, the prevalence of giardiasis in patients with diarrhoea is about 20%, (range 5–43%) (Islam 1990). The figure for developed countries varies from 3% (Hoogenboom-Verdegaal et al., 1989; Adam, 1991; Farthing, 1994; Kortbeek, van Deursen & Hoogenboom-Verdegaal, 1994) to 7% (Quinn, 1971).

Both humoral and cellular immune responses are elicited by infection with *Giardia*. Secretory IgA and IgM appear to play a role in clearance of the intestinal

infection, by reducing the mobility of trophozoites and preventing their adhesion to the mucosa (Farthing & Goka, 1987). The immune response can also be seen in the serum antibodies. The immune response provides some protection against reinfection, as indicated by lower attack rates in chronically exposed populations (Istre et al., 1984; Rabbani & Islam, 1994). This protection is limited, however, and recurrence of symptomatic infections is common, even after several infections (Gilman et al., 1988; Wolfe 1992; Hall, 1994), which may be related to the antigenic variation shown by *Giardia* (Nash, 1992).

Giardiasis can be treated with nitroimidazoles, quinacrine, and furazolidone (Boreham, 1994). For patients with persistent giardiasis several approaches are possible, such as increasing the doses and duration of treatment, or giving an alternative drug or a combination of drugs.

Routes of transmission

Faecal–oral transfer of *Giardia* cysts is the major route of transmission of giardiasis, as indicated by the high prevalence in developing countries with poor standards of hygiene and sanitation, in day-care centres and nurseries (Black et al., 1977; Pickering & Engelkirk, 1990; van de Bosch, 1991), and by secondary spread within the household of those who attend day-care centres (Black et al., 1977). Foodborne outbreaks are the result of contamination of food by infected workers or household members (Osterholm et al., 1981; Islam, 1990; Thompson, Lymbery & Meloni, 1990).

The role of animals in the transmission of human giardiasis is still a matter of some speculation. Although *Giardia* commonly occurs in domestic pets, farm animals, and wild mammals, there is no unequivocal evidence that organisms from these sources have caused infections in humans (Erlandsen, 1994). *Giardia intestinalis* isolates from animals and humans may be morphologically indistinguishable (Flannagan, 1992) and this has led to many reports of animal sources of human giardiasis, including waterborne infections caused by *Giardia* cysts from beavers and muskrats (Moore et al., 1969; Dykes et al., 1980). However, the genetic diversity within and between human and animal isolates (Thompson, Meloni & Lymbery, 1988) is too high to allow definite conclusions to be drawn regarding host specificity. Cross-transmission studies have not been well controlled and the results have been contradictory (Davies & Hibler, 1979; Hewlett et al., 1982; Belosevic, Faubert & MacLean, 1984; Kirkpatrick & Green, 1985; Woo & Patterson, 1986).

Waterborne outbreaks of giardiasis have been reported for some 30 years (Moore et al., 1969; Brodsky, Spencer & Schultz, 1974; Craun, 1990). In the USA, *Giardia* is the most commonly identified pathogen in outbreak investigation, with more than 100 waterborne outbreaks, based on epidemiological evidence (Craun, 1990). Waterborne outbreaks have also been reported in Australia, Canada, New Zealand, Sweden, and the United Kingdom. These outbreaks have

been linked to consumption of untreated surface water contaminated by human sewage (Craun, 1990) or by wild rodents (Moore et al., 1969; Dykes et al., 1980), to groundwater that was contaminated by human sewage or contaminated surface water, to surface water systems treated only by disinfection (Craun, 1984; Kent et al., 1988) or by ineffective filtration (Dykes et al., 1980; Craun, 1990), and to cross-connections or damage in water-distribution systems (Craun, 1986).

Cyclospora cayatanensis

Taxonomy

Cyclospora was first isolated by Eimer in 1870 from the intestines of moles; it is related taxonomically to other protozoan parasites such as *Cryptosporidium* and *Toxoplasma*. The first observation of this parasite as a pathogen for human beings was probably that reported by Ashford (1979). Confirmation of the coccidian identity and genus was made in 1993 (Ashford et al., 1993; Ortega et al., 1993). *Cyclospora* is a member of the subphylum Apicomplexa, class Sporozoa-sida, subclass Coccidiasina, family Eimeriidae. Molecular phylogenetic analysis suggests that the genus is closely related to the genus *Eimeria* (Relman et al., 1996). *Cyclospora* organisms have been found in snakes, insectivores, and rodents.

Life cycle

Cyclospora completes its life cycle within one host (monoxenous), but many details remain to be elucidated. Ortega et al. (1993) proposed that *Cyclospora* that are infective to human beings should be designated *Cyclospora cayatanensis* on the basis of the development of the oocyst in vitro. However, use of this species name was questioned by Ashford, Warhurst & Reid (1993), and Bendall et al. (1993) prefer the term CLB (for *Cyclospora*-like body) until further information is available on the biology of this coccidian parasite. For the purposes of this review, however, the term "*Cyclospora* spp" is used to describe organisms of this genus that are infective to humans.

The endogenous stages of *Cyclospora* sp. are intracytoplasmic and contained within a vacuole (Bendall et al., 1993), and the transmissive stage, the oocyst, is excreted in the stool. The life cycle of *Cyclospora* sp. may complete within enterocytes (Sun et al., 1996). *Cyclospora* sp. oocysts are spherical, measuring 8–10 µm in diameter; they are excreted in the stool and sporulate to infectivity in the environment. Unsporulated oocysts contain a central morula-like structure consisting of a variable number of inclusions. Sporulated oocysts contain two ovoid sporocysts, within each of which there are two sporozoites (Levine, 1973). Each sporozoite measures $1.2 \times 9 \mu\text{m}$.

Pathogenicity

Cyclospora sp. infect enterocytes of the small bowel and can produce disease (Bendall et al., 1993). Both symptomatic and asymptomatic states have been described. A moderate to marked erythema of the distal duodenum can occur with varying degrees of villous atrophy and crypt hyperplasia (Connor et al., 1993), but little is known of the pathogenic mechanisms. As yet, no virulence factors have been described for *Cyclospora* sp. No animal or human feeding studies have been undertaken. As for *Giardia* and *Cryptosporidium*, it is assumed that the organisms are highly infectious, and that doses lower than 100 sporulated oocysts carry a high probability of infection.

The disease

Symptoms of infection include watery diarrhoea, fatigue, abdominal cramping, anorexia, weight loss, vomiting, low-grade fever, and nausea; these can last for several weeks, with bouts of remittance and relapse. The incubation period is between 2 and 11 days (Soave, 1996) with moderate numbers of unsporulated oocysts being excreted for up to about 60 days. Illness may last for weeks and episodes of watery diarrhoea may alternate with constipation (Soave, 1996). In immunocompetent individuals the symptoms are self-limiting and oocyst excretion is associated with clinical illness (Shlim et al., 1991); in immunocompromised patients, diarrhoea may be prolonged.

Prevalence

Oocysts of *Cyclospora* sp. have been isolated from the stools of children and from both immunocompetent and immunocompromised adults. They have been described in the stools of residents of, and travellers returning from, developing countries, and in association with diarrhoeal illness in individuals from north, central, and south America, south-east Asia, Australia, the Caribbean, Europe, and the Indian subcontinent. Outbreaks of cyclosporiasis have been reported from north and south America and from Nepal. In north America and Europe cyclosporiasis is associated with overseas travel and travellers' diarrhoea. Point-source outbreaks have been reported in Nepal and the USA. In 1996, a total of 1465 cases were reported in Canada and the USA; most cases occurred during spring and summer, and about half occurred following events at which raspberries had been served (Centers for Disease Control and Prevention, 1996; Herwaldt & Ackers, 1997). Sporadic cases of cyclosporiasis have been reported from many countries and *Cyclospora* sp. oocysts are increasingly being identified in stools from immunocompetent individuals with no history of foreign travel.

Cyclospora sp. oocysts were detected in faecal samples from 11% of Haitians with chronic diarrhoea who were HIV-seropositive (Pape et al., 1994); they were the only pathogens, other than HIV, identified in many of these patients. Clin-

ical disease may resolve without treatment, but trimethoprim–sulfamethoxazole is the treatment drug of choice.

Routes of transmission

Epidemiological data indicate that *Cyclospora* spp are transmitted by water and food (Hoge et al., 1993; Centers for Disease Control and Prevention, 1996; Herwaldt & Ackers, 1997). An outbreak occurred among house staff and employees in a hospital dormitory in Chicago following the failure of the dormitory's water pump. Illness was associated with the ingestion of water in the 24 hours after the pump failure, and *Cyclospora* spp oocysts were detected in the stools of 11 of the 21 individuals who developed diarrhoea (Centers for Disease Control, 1991; Wurtz, 1994).

In an outbreak that occurred among British soldiers and their dependants stationed in a small detachment in Nepal, 12 people out of 14 developed diarrhoea. *Cyclospora* oocysts were detected in stool samples from 6 of 8 patients. Oocysts were also detected microscopically in a concentrate from a 2-litre water sample. Drinking-water for the camp consisted of a mixture of river water and chlorinated municipal water. Chlorine residuals of 0.3 to 0.8 ppm were measured before and during the outbreak. No coliforms were detected in the drinking-water (Rabold et al., 1994).

Monitoring and assessment

Cryptosporidium and *Giardia*

The methodology for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in water is completely different from that traditionally used for quantification of faecal indicator bacteria in the water industry. Currently available methods are at best tentative—recovery is low and variable, and it is not possible to differentiate viable oocysts of strains that are infectious to humans. The procedure consists of several stages: sample collection and concentration, separation of (oo)cysts from contaminating debris, and detection of (oo)cysts. Many factors, such as water quality and age of the (oo)cysts, can have significant effects on the overall efficiency of recovery, and it is almost impossible to compare the effectiveness of methods used in different laboratories unless these factors are standardized.

There is considerable interest in determining whether (oo)cysts recovered from the environment are viable and potentially infectious.

Quality assurance

Microscope counts. Care must be taken to ensure that the particles being counted are (oo)cysts, to determine whether or not they contain sporozoites, and to exclude algae and yeast cells from any counts that are made. The criteria used for

determining that a particle is in fact a *Cryptosporidium* oocyst or a *Giardia* cyst vary between laboratories. Some workers use only the fact that (oo)cysts fluoresce when labelled with a fluorescein isothiocyanate-tagged anti-*Cryptosporidium* or anti-*Giardia* monoclonal antibody and that it is in the proper size range for a cyst or oocyst. Others will additionally use differential interference contrast microscopy or nucleic acid stains to ascertain that the particles counted are indeed (oo)cysts. This more detailed analysis allows the confirmation of the counted particles as presumptive (oo)cysts.

Many factors influence the microscope counts: the amount of background debris and background fluorescence, the experience and alertness of the technician who performs the count, the intensity of fluorescence after staining with the monoclonal antibody, and the quality of the microscope. Quality assurance protocols should define how these factors are addressed.

Recovery efficiency. In view of the low and variable efficiency of recovery in the methods used for monitoring *Cryptosporidium* and *Giardia*, it is essential that laboratories collect their own data on recovery efficiency in the different water types they monitor. This can be achieved by seeding a second water sample with a known number of cysts and oocysts and determine the percentage of these recovered by the total protocol for sampling, processing, and counting of environmental samples. However, this assay is influenced by the number, age, and storage conditions of the seeding (oo)cysts, all of which should be standardized (at least within a particular laboratory) if recovery data are to be meaningful. The recovery efficiency should be assessed sufficiently often to reveal how its variation influences the uncertainty of the monitoring data. This is essential for the interpretation of environmental monitoring data.

Cartridge filtration. The first reported method for detection of *Giardia* and *Cryptosporidium* in water used polypropylene cartridge filters, with a nominal pore size of 1 μm , through which large volumes of water (100–1000 litres) were passed at a flow rate of 1–5 litres/minute. Trapped material was then eluted by cutting open the filter and washing it either by hand or by stomaching using a dilute detergent solution. The resulting washings from these cartridges sometimes totalled 3 or 4 litres and required further concentration by centrifugation. The recovery of *Cryptosporidium* oocysts by this technique was originally reported to be 14–44% (Musial et al., 1987), although lower efficiencies (<1–30%) have often been reported since then (Ongerth & Stibbs, 1987; Clancy, Gollnitz & Tabib, 1994; Shepherd & Wyn-Jones, 1996). Differences in reported recovery rates may be due to a number of factors including water quality, laboratory efficiency, and oocyst age.

Membrane filtration. Ongerth & Stibbs (1987) described a method using large (diameter 142 or 293 mm), 2 μm absolute, flat-bed membranes for the concentration of oocysts from water samples, and many workers have now adopted

this procedure. Water is pumped through the membranes and the concentrated materials are recovered by "scraping" the surface of the membrane together with washing with dilute detergent, followed by further concentration using centrifugation. However, while it is relatively easy to filter 10–40 litres of low-turbidity water, with some high-turbidity waters it is possible to filter only 1–2 litres. As with cartridge filtration, a range of recovery efficiencies has been reported for flat-bed membranes. Nieminski, Schaeffer & Ongerth (1995) reported an average recovery of 9% for *Cryptosporidium* and 49% for *Giardia*. In a study of the recovery efficiency of several different membranes, Shepherd & Wyn-Jones (1996) suggested that 1.2- μm cellulose-acetate membranes gave higher recovery (30–40% and 50–67%, respectively, for *Cryptosporidium* and *Giardia*) than the 2- μm polycarbonate membranes (22–36% and 41–49% respectively) preferred by Ongerth & Stibbs (1987).

Flocculation. Another established method for concentrating (oo)cysts is the calcium carbonate flocculation procedure developed by Vesey et al. (1993b). A fine precipitate of calcium carbonate (CaCO_3) is formed in a water sample by adding calcium chloride and sodium bicarbonate and adjusting the pH to 10.0 with sodium hydroxide. After the precipitate has settled, the supernatant fluid is removed by aspiration, the calcium carbonate is dissolved with sulfamic acid, and the sedimented material is resuspended. Recovery efficiencies using this method have been reported to be as high as 70% for both *Cryptosporidium* and *Giardia* (Vesey et al., 1993b; Campbell et al., 1994; Vesey et al., 1994; Shepherd & Wyn-Jones, 1996). More recent work has demonstrated that this is the upper limit of the detection efficiency and that recoveries are usually lower. Using aged oocysts for seeding experiments and leaving the oocysts in contact with water for a few days before analysis normally produces recovery rates of 30–40%. The viability of the oocysts is affected by this concentration (Campbell et al., 1995). Flocculation with aluminium sulfate ($\text{Al}_2(\text{SO}_4)_3$) does not affect the viability of oocysts, while the recovery efficiency is comparable to that achieved with CaCO_3 flocculation (Schwartzbrod, personal communication).

New methods. The search continues for new methods of concentrating water samples to detect the presence of protozoan parasites, and many techniques have been evaluated, including cross-flow filtration, continuous-flow centrifugation, and vortex-flow filtration (Whitmore, 1994) as well as a number of proprietary systems.

There continues to be much debate over which method is most appropriate. Realistically no single method is suitable for all situations. The choice of method should be made with due regard to a number of factors, including the purpose of sampling, the water quality, and the facilities in the laboratory that will perform the analysis. Ideally, the method chosen should efficiently concentrate as large a sample as possible and yield a concentrate that can be examined easily. Many workers prefer to concentrate only a small volume of water and to examine the entire concentrate, while others take large samples and examine only a

fraction of the final concentrate. Both approaches are valid, but the methods used to concentrate small volumes (e.g. 10–20 litres) tend to be easier to perform and generally have a higher recovery efficiency. It is therefore often preferable to take a large number of low-volume samples and examine all of the concentrate. Other factors that may affect the choice of concentration method include the site of sample collection and the distance over which samples must be transported.

Separation techniques

Since the concentration of *Cryptosporidium* oocysts and *Giardia* cysts is based almost exclusively on particle size, the techniques are not specific and a large amount of extraneous material is concentrated as well. This material may interfere with the successful detection of (oo)cysts, either by increasing the total volume of material that needs to be examined, or by obscuring or mimicking (oo)cysts during examination. Some form of separation technology is therefore normally required to reduce the time taken to examine a sample and to prevent (oo)cysts being missed.

Density centrifugation. Density centrifugation is often used to separate (oo)cysts from background debris and thus reduce the amount of material to be examined. Several workers use sucrose density centrifugation to separate parasites from faecal material in clinical samples, and this basic technique has also been adopted for use with environmental samples. Whatever flotation method is used, several groups have demonstrated that this procedure is inefficient for detecting protozoan parasites in water concentrates. Of particular interest was the finding of Bukhari & Smith (1996) that sucrose density centrifugation selectively concentrated viable, intact *Cryptosporidium* oocysts. Fricker (1995) demonstrated that the recovery of oocysts from water samples could be affected by the length of time that they were in contact with the water concentrate but only when sucrose flotation was used. Spiked samples examined directly, without density centrifugation, gave similar recovery efficiencies, whether they were examined immediately after seeding or after 48 hours' contact with the concentrate. When sucrose flotation was used, however, the recovery of (oo)cysts in raw water fell from a mean of 55% to 18% after the same period of contact. This reduction in recovery efficiency also occurred with concentrates of reservoir water (67% to 23%) and fully treated water (80% to 52%).

Immunomagnetic separation. Autofluorescing algae, which may not be completely removed by density gradient centrifugation, can cause severe problems when slides are examined for protozoa by epifluorescence microscopy. More efficient methods for separation of (oo)cysts from other particulates have been sought, and many workers have tried immunomagnetic separation. The principles underlying this technique are attachment of specific antibodies to magnetizable

particles and efficient mixing of the particles in the sample. The (oo)cysts attach to the magnetizable particles and are isolated from this debris with a strong magnet. The technique is very simple, but there are several sources of failure, including the quality and specificity data of the available monoclonal antibodies. Most of the commercially available monoclonal antibodies to *Cryptosporidium* or *Giardia* are of the IgM type, and are therefore of low affinity since they have not undergone affinity maturation or isotype switching. When immunomagnetic separation is used and beads are mixed with water concentrates, the immunoglobulin-(oo)cyst bonds are subjected to shear forces; the stronger the bond, the more likely the bead is to remain in contact with the (oo)cyst. The way in which the antibody is attached to the bead may also have an effect on recovery efficiency: if the attachment is weak, the antibody may detach and the oocyst will not be recovered.

The turbidity of the water concentrate appears to be the most critical factor associated with the recovery efficiency of immunomagnetic separation. Oocysts seeded into relatively clean suspensions are recovered efficiently—Campbell & Smith (1997) and Campbell, Gron & Johnsen (1997) have reported recoveries in excess of 90%. However, the real benefit of a good separation technique is seen with samples that have yielded a highly turbid concentrate; in these samples immunomagnetic separation seems to perform less efficiently. The use of antibodies of higher affinity may improve the recovery efficiency of oocysts from high turbidity samples. Although the technique is also able to separate *Giardia* cysts, little effort has been devoted to testing the recovery efficiency of these cysts by immunomagnetic separation.

Flow cytometry. Flow cytometry has been attempted with environmental samples to detect *Cryptosporidium* oocysts, but it was found that the sensitivity of the instruments was insufficient to distinguish oocysts from background noise (Vesey et al., 1991). However, incorporation of a cell-sorting facility enabled oocysts to be sorted efficiently from background material (Vesey et al., 1993a). This technique seems to work equally well for *Giardia* cysts (Vesey, Slade & Fricker, 1994; Medema et al., 1998a). Water concentrates are stained in suspension with fluorescein isothiocyanate-labelled (FITC-labelled) antibodies and passed through the fluorescence-activated cell sorter (FACS). Particles with the fluorescence and light-scattering characteristics of (oo)cysts are sorted from the sample stream and collected on a microscope slide or membrane filter, which is then examined by epifluorescence microscopy to confirm the presence of (oo)cysts. The FACS procedure is not sufficiently specific or sensitive for the count of sorted particles to give a definitive indication of the number of (oo)cysts present. Other organisms and particles of similar size may cross-react with the monoclonal antibody and have similar fluorescence characteristics. Moreover, some water samples contain high numbers of autofluorescent algae which may also mimic (oo)cysts and lead to incorrect conclusions if the FACS is used directly to produce (oo)cyst counts. However, the confirmation by epifluorescence microscopy can be performed

much more easily and reliably than direct microscopy of non-sorted samples. Several researchers from France, the Netherlands, and the United States have confirmed the benefits of FACS for examining water samples for the presence of (oo)cysts (Danielson, Cooper & Riggs, 1995; Compagnon et al., 1997; Medema et al., 1998a). FACS is widely used in the United Kingdom for water analysis and is becoming more widely adopted in other parts of Europe, in Australia, and in South Africa.

Detection

Immunofluorescence microscopy. Detection of *Cryptosporidium* oocysts and *Giardia* cysts relies on epifluorescence microscopy, which may be used to examine material deposited on multi-well slides or membrane filters. The (oo)cysts are specifically stained with monoclonal antibodies which have been either labelled directly with FITC or labelled during staining with an FITC-labelled anti-mouse antibody. There have been no definitive studies to compare the efficiency of these procedures, but the tendency now is towards staining with a directly labelled antibody, which seems to produce less nonspecific binding and can make preparations easier to examine. Several anti-*Cryptosporidium* and anti-*Giardia* antibodies are commercially available; most workers have their own preferences, and there does not appear to be a single antibody that is preferred for all purposes. A failing of some antibodies, including commercially available antibodies, is that they apparently cross-react with other members of the genera and therefore cannot be used to specifically identify *C. parvum* or *G. intestinalis*.

A number of other detection techniques have been tried in an effort to improve the ease of identification of both *Cryptosporidium* oocysts and *Giardia* cysts.

Fluorescence in-situ hybridization (FISH). FISH has been suggested as a tool for the specific detection of *Cryptosporidium parvum* (Lindquist, 1997; Vesey et al., 1997). Vesey et al. (1997) also showed that the stainability of oocysts with the FISH method correlated with excystation. The FISH method could be combined with the immunofluorescent assay (IFA) method. However, the FISH-fluorescence signal is relatively weak, which makes microscopic interpretation difficult.

Polymerase chain reaction (PCR). One of the most extensively tested procedures is the polymerase chain reaction for detection of specific sequences of nucleic acids that may be species- or genus-specific. Clearly, the ability to distinguish between *C. parvum* and other morphologically similar members of the genus is useful and nucleic-acid-based techniques may prove useful for this.

Despite the specificity and sensitivity offered by PCR, however, difficulties have been experienced with application of the technique to water concentrates, largely because of inhibition of the DNA amplification process. PCR is sensitive to the concentration of many compounds within the reaction mixture; those of

particular concern to researchers working with water concentrates are divalent cations and humic and fulvic acids, which are frequently found in water and can cause a high degree of inhibition. Nonetheless many workers have described protocols for the detection of *Cryptosporidium* oocysts by PCR and a wide variety of primers have been described. These primers have been designed from various regions of the genome. Primers with apparent specificity include those from regions coding for the 18S rRNA (Johnson et al., 1995), or mRNA coding for the *Cryptosporidium* heat shock protein *Hsp70* (Stinear et al., 1996, Kaucner & Stinear, 1998), in combination with cell culture (Rochelle et al., 1996; Rochelle, 1997).

Abbaszadegan et al. (1997) first reported the use of PCR primers from gene sequences coding for inducible heat shock proteins to specifically detect *Giardia* cysts. The sensitivity of the standard PCR was reported to be one cyst in water samples. These workers also reported that amplification of heat-shock-induced mRNA using the same HSP primers was indicative of viable *Giardia* cysts.

The use of PCR for the detection of (oo)cysts in water concentrates offers some advantages over direct microscopic examination, since the procedure can be largely automated, allowing several samples to be handled simultaneously. Furthermore, the technique is, theoretically, sensitive down to the level of a single (oo)cyst, and recent developments have suggested that it may be possible to distinguish viable from non-viable (oo)cysts. Some workers claim to be able to detect a single oocyst in a water concentrate by using a procedure involving reverse transcription (RT-PCR) where the target sequence codes for the *Cryptosporidium* heat shock protein *Hsp 70* (Stinear et al., 1996). The data presented showed that a single viable oocyst could be detected even in the presence of PCR inhibitors. Such a method would be of considerable value to the water industry, facilitating rapid screening of samples; as yet, though, the method is not quantitative and thus may be of limited value in some circumstances.

The use of RT-PCR against induced mRNA, a nucleic acid with a short half-life, overcomes the concern that false-positive results could be obtained either from non-viable oocysts or from free DNA. Many researchers still favour a holistic approach, where the intact organism can be viewed directly. A combined approach might be possible, with molecular techniques being used as a screening tool on a portion of a water concentrate, followed by microscopic examination when positive results are obtained.

Methods for determining oocyst viability

The significance of finding oocysts in treated and, to a lesser extent, raw waters is not always clear, since some of the organisms that are detected may be non-viable and thus pose no threat to public health. Consequently, there has been considerable interest in developing in vitro methods capable of determining oocyst viability.

Excystation. The most widely accepted in vitro procedure for determining oocyst viability, excystation, has not been used with the IFA method, because it is difficult to incorporate into the IFA protocol. However, it has been used in combination with PCR to detect the presence of viable *Cryptosporidium* oocysts (Filkorn, Wiedenmann & Botzenhart, 1994; Wiedenmann et al., 1997). The sensitivity of this method in environmental samples needs further research. Excystation has also been used in survival and disinfection studies; in the latter, the technique appears to yield a lower inactivation rate than the neonatal mouse infectivity assay (Finch et al., 1993a; Clancy et al., 1998).

Vital dyes. The ability of *Giardia* cysts to stain with the vital exclusion dye propidium iodide (PI) has been shown by various workers to correlate with their inability to excyst or infect animals (Schupp & Erlandsen, 1987; Smith & Smith, 1989). It is therefore possible to use PI as an indicator of cell death for *Giardia* cysts.

Campbell, Robertson & Smith (1992) developed a procedure for *Cryptosporidium* oocysts based on the exclusion of PI; they used 4'-diamidino-2-phenyl indole (DAPI) as supporting stain, which gave a good correlation with in vitro excystation. Four classes of oocysts can be identified using the assay: those that are viable and include DAPI but exclude PI, those that are non-viable and include both DAPI and PI, and two classes that include neither DAPI nor PI—those with internal contents (sporozoites) and therefore potentially viable, and those without and therefore non-viable, as determined by differential interference contrast (DIC) microscopy. The DAPI/PI procedure is simple to perform and, despite some workers' reservations about its applicability, can be used for routine environmental work. The incorporation of DAPI into the nucleic acid acts as a further criterion for determining whether a particle is an oocyst or not.

An alternative to the DAPI/PI approach for determining viability has been suggested by Belosevic & Finch,¹ who used new nucleic acid stains to differentiate between viable and non-viable oocysts. Two new stains have been identified. With SYTO9, non-viable oocysts stain green or bright yellow; a viable oocyst has a green halo but its interior remains unstained. MPR71059 stains non-viable oocysts red while viable oocysts remain unstained. These methods have not been widely tested, although Belosevic & Finch (1997) demonstrated that the results obtained with the dyes correlate well with mouse infectivity using an outbred CD-1 neonatal mouse model. Since these vital-stain assays are apparently simple and quick to perform, they may be suitable for incorporation into the methods for the detection of oocysts in water samples—but this has yet to be proven.

Cell culture. Attempts have been made to develop in vitro models of infectivity using tissue culture (Upton, Tilley & Brillhart, 1994; Rochelle et al., 1996; Slifko,

¹ Presented at the International Symposium on Waterborne *Cryptosporidium*, March 1997, Newport Beach, CA, USA.

1997). For these assays, water samples are concentrated by normal procedures and bacteria may be removed by exposure of the concentrate to chlorine at levels that are lethal to bacterial cells but that are thought not to affect oocysts. The concentrates are then inoculated onto the tissue-culture monolayer and left in contact for a period to allow potentially infectious oocysts to infect cells before the remaining debris is washed away. The monolayer is then left for 24–48 hours before being examined for the presence of intracellular parasite antigen or nucleic acid. Immunofluorescent techniques have been used to identify cells that have become infected. This method offers a means of quantifying infection, although it is not clear whether the presence of a single infectious oocyst will lead to one or more infected cells. In theory, an oocyst that excysts successfully would be expected to produce 2–4 infected tissue culture cells (for *Giardia* and *Cryptosporidium* respectively), but initial results have not demonstrated that this can be consistently achieved.

Some workers (Rochelle et al., 1996) have adopted a somewhat different approach, detecting the presence of *Cryptosporidium* nucleic acids using PCR. While the cell culture method cannot be used to directly enumerate the oocysts present in any given sample, it can be applied in a “most probable number” format to give an estimate of the number of oocysts present in a water concentrate.

Molecular methods. The RT-PCR methods that amplify induced mRNA coding for heat shock proteins can also be used to indicate viability of *Giardia* cysts (Abbaszadegan et al., 1997) and *Cryptosporidium* oocysts (Stinear et al., 1996; Kaucner & Stinear, 1998). In combination with the reported sensitivity and specificity (see p. 89), these methods may in the future prove to be very valuable for the water industry.

Typing methods

With the current detection techniques, it is not possible to identify the origin of (oo)cysts in a water sample. Several typing methods are available for both *Cryptosporidium* and *Giardia* and will discriminate between human and animal *C. parvum* strains (Ogunkolade et al., 1993; Bonnin et al., 1996; Deng & Cliver, 1998); however, these methods are not yet applicable to surface-water samples.

Cyclospora

Detection methods for stool samples

No methods have been developed for the detection of *Cyclospora* in environmental samples; the information provided in this section on detection of the parasite in stool samples is therefore intended for guidance only.

Identification of *Cyclospora* in stool samples is based on the appearance of the oocyst in either direct or concentrated wet films. Concentration either by the formalin–ether (formalin–ethyl acetate) method or by sucrose flotation is effective. Oocysts have also been reported from jejunal aspirates (Bendall et al., 1993). Organisms seen in stool samples are normally the unsporulated oocysts of *Cyclospora* sp. By bright-field microscopy of wet mounts, oocyst walls appear as well-defined, non-refractile spheres of remarkably uniform size (diameter 8–10 µm) (Ashford, 1979; Long et al., 1991); within each oocyst is a central morula-like structure containing a variable number of inclusions. At higher (×400) magnification, the inclusions appear refractile, exhibiting a greenish tinge. Oocysts that are empty or that have collapsed into crescents are occasionally seen. Under UV illumination (330–380 nm) the oocyst wall autofluoresces so that the organisms appear as blue circles.

The organisms do not stain with Lugol's iodine. Staining of air-dried faecal smears with acid-fast stains can aid identification, and, according to Wurtz (1994), the rapid dimethyl sulfoxide-modified acid-fast staining method is more effective than either the Kinyouin or the modified Ziehl-Neelsen method. Oocysts stain variably with acid-fast stains, from no staining through to deep red. A modified safranin method (microwaving followed by safranin staining) stains oocysts a brilliant reddish orange (Visvesvara et al., 1997).

Sporulated oocysts contain two sporocysts and each sporocyst contains two crescent-shaped sporozoites. In instances where excystation has been achieved in vitro, by exposure of oocysts/sporocysts to an excystation medium at 37 °C for up to 40 minutes, two sporozoites emerge from each sporocyst.

Concentration techniques for environmental samples

No method has been developed specifically for the detection of *Cyclospora* sp. in environmental samples. However, *Cyclospora* sp. oocysts are larger than *C. parvum* oocysts and smaller than *G. intestinalis* cysts, and it is therefore assumed that methods developed for *Cryptosporidium* and *Giardia* will prove effective for sampling and recovering *Cyclospora* sp. oocysts from water concentrates.

Detection techniques for use in environmental samples

There are no in vitro culture methods for increasing the numbers of *Cyclospora* sp. oocysts nor have any in vivo amplification models been described. A proportion of oocysts stored in faeces, water, or 2.5% potassium dichromate at temperatures between 22 °C and 37 °C for up to 14 days in the laboratory will sporulate (Ortega et al., 1993; Smith et al., 1997). Currently there is no commercially available polyclonal or monoclonal antibody with specificity to exposed epitopes on *Cyclospora* sp. oocysts; the autofluorescent properties of the oocyst wall under UV illumination have therefore been used in an attempt to detect

oocysts in a variety of food and water concentrates. The primers identified by Relman et al. (1996), which amplify the small subunit rRNA coding region, have been used to amplify the *Cyclospora*-specific sequence from nucleic acid liberated from the berries (strawberries and raspberries) implicated in a series of outbreaks in the USA in 1996. To date, however, no positive results have been reported.

Control

Cryptosporidium and *Giardia* are ubiquitous in surface waters worldwide. Reported concentrations are generally in the range 0.01–100 per litre; these values are not corrected for the (low) recovery of the detection method, so actual concentrations may be more than 10-fold higher. Higher concentrations are found in urban or agricultural waters than in pristine waters (LeChevallier, Norton & Lee, 1991; Rose, Gerba & Jakubowski, 1991).

Sources of surface-water contamination are the discharge of untreated and treated sewage, manure, run-off from grazing land, and wildlife. The relative significance of these sources may differ between watersheds. Large rivers and lakes often receive both agricultural run-off and treated and untreated domestic wastewater, and their relative contribution has not been quantified. Contamination by wildlife may be important in pristine watersheds and has been implicated as a source of waterborne giardiasis, although this is still a matter of much controversy.

Oocysts and cysts can survive for months in surface water (DeReignier et al., 1989; Robertson, Campbell & Smith, 1992; Chauret et al., 1995; Medema, Bahar & Schets, 1997). Under natural conditions, the die-off rate of *Cryptosporidium* oocysts in water is 0.005–0.037 log₁₀-units per day. For *Giardia*, the die-off rate is higher and more temperature-dependent, varying between 0.015 log₁₀-units per day at 1 °C and 0.28 log₁₀-units per day at 23 °C (DeReignier et al., 1989). Little information is yet available on the significance to the environmental ecology of the state in which (oo)cysts occur in water, i.e. suspended or attached to particles (although this is relevant for water treatment by sedimentation and filtration), or the fact that they readily attach to particles (Medema et al., 1998b).

Recent work showed that, overall, 12% of groundwater supplies in the US were contaminated with *Cryptosporidium* and/or *Giardia* (Hancock, Rose & Callahan, 1997), mostly in infiltration galleries and horizontal wells. No data on the level of protection and travel time and distance of these groundwater sources were given.

Prevention of the transmission of protozoan parasites through drinking-water requires a multiple barrier approach: protection of watersheds used for drinking-water production against contamination with protozoa, plus adequate treatment of water—and verification by monitoring of water quality and operational parameters that the treatment is effective.

Watershed protection

One of the most important aspects of watershed protection is the recognition of local sources of contamination with *Cryptosporidium* and *Giardia* and the control of that contamination by diversion or treatment of discharges and reduction of direct input of faeces, especially in otherwise pristine waters, by people, farm animals, and wildlife or from manure storage. Treatment of sewage in activated sludge systems or waste stabilization ponds is an important barrier against environmental transmission: both processes remove 90–99.7% of the cysts and oocysts (Sykora et al., 1991; Grimason et al., 1992).

Treatment of agricultural wastes before their application to the land also reduces the number and viability of *Cryptosporidium* oocysts: aerobic treatment of cattle slurry at increased temperatures and ammonia concentrations rapidly inactivates oocyst (Svoboda et al., 1997) and composting of bedding reduces the viability of oocysts.

Storm run-off and snowmelt from unprotected watersheds have been implicated as sources of peak contamination of source water (Stewart et al., 1997; Atherholt et al., 1998), and may result in treatment overload and the contamination of drinking-water with (oo)cysts. Knowledge of the characteristics of the plume of contamination from watershed sources can be used to locate and design abstraction points. An illustration of the importance of this is provided by the intake of the southern plant of Milwaukee in Lake Michigan, which proved to be exactly in the plume of the Milwaukee river. The turbidity in the raw water peaked and this coincided with treatment failure, resulting in the breakthrough of turbidity and oocysts into Milwaukee drinking-water and a consequent massive outbreak of disease (MacKenzie et al., 1994). Installation of pretreatment storage reservoirs flattens peak contaminations (Ketelaars et al., 1995) and the storage capacity makes it possible to stop the intake of surface water temporarily during high contamination events (see “Pretreatment reservoirs”, page 96).

Since the protozoa are typically related to faecal contamination of surface water, several studies have investigated the use of indicator bacteria to predict high levels of protozoa. No consistent relationship has been observed, however, between indicator bacteria (thermotolerant coliform) levels and concentrations of *Giardia* or *Cryptosporidium*. The low and varying recovery rates of the protozoa detection methods may be an important confounder in detecting these relationships. Since (oo)cysts are much more persistent than coliforms and enterococci in water, it is likely that these bacteria are not valid indicators, especially if the contamination source is distant. More persistent bacterial indicators (spores of *Clostridium perfringens*) may prove useful indicators for these protozoa (Payment & Franco, 1993; Hijnen et al., 1997). In the absence of valid surrogates, watershed assessment to determine local sources of contamination and define the amount of treatment necessary should include monitoring for protozoa.

Development of transport and fate models for predicting (oo)cyst concentrations based on data on the sources may help in identifying important sources

or environmental events that determine protozoa levels at abstraction points (Medema et al., 1997).

The number of species of *Cyclospora* that are infective to human beings is not currently known, nor is it known whether human-derived oocysts are infectious to non-human hosts. However, the primary sources of contamination will be human faeces containing oocysts. Because the oocysts of *Cyclospora* sp. are larger than those of *Cryptosporidium parvum* but smaller than *G. intestinalis* cysts, it is likely that they will be discharged with final effluents from waste stabilization ponds and sewage treatment works. Oocysts take up to 14 days to mature (sporulate) in the laboratory, but sporulate more rapidly at higher (up to 37°C) temperatures. Sporulation time in the environment will thus depend upon ambient temperature, and sporulated oocysts may be found distant from the pollution source in the aquatic environment. Sources of unsporulated oocysts are likely to be effluent discharges from sewage treatment and waste stabilization ponds with detention times of less than 1 week.

Like *C. parvum* oocysts and *G. intestinalis* cysts, oocysts of *Cyclospora* sp. are likely to survive longer at lower temperatures when suspended in water. Oocysts stored at 4°C do not appear to sporulate (Smith et al., 1997); however, a proportion of oocysts stored for up to 2 months at 4°C will sporulate when subsequently incubated at temperatures between 22°C and 37°C. No data are available regarding survival and transport in soil.

Adequate treatment

Filtration

The principal barrier for protozoa is physical removal by filtration. *Cryptosporidium* oocysts are relatively small, making them more difficult to remove than *Giardia* cysts. Rapid sand filtration, a common treatment process used to remove particles, is theoretically capable of 3 log removal of *Cryptosporidium* oocysts (Ives, 1990). Other investigators have published a range of removal rates, from 91% (Rose et al., 1986) to greater than 99.999% (Hall, Pressdee & Carington, 1994); the higher removal rates were achieved when coagulant dosing was applied to the water before filtration.

Diatomaceous earth filtration has been reported to remove more than 99% of *Giardia* cysts (Jakubowski, 1990) and up to 4–6 log-units for *Cryptosporidium* under laboratory conditions (Ongerth & Hutton, 1997).

Conventional treatment (coagulation, sedimentation, filtration), direct filtration (with chemical pretreatment), and high-rate filtration can remove 99% of (oo)cysts when systems are properly designed and operated (LeChevallier, Norton & Lee, 1991; Nieminski, 1994; West et al., 1994). Typically the chemicals used are ferric or aluminium salts and there appears to be no real difference in the effectiveness of aluminium sulfate, polyaluminium chloride, ferric sulphate, and ferric chloride in removing oocysts and other particles of similar size (Ives, 1990).

If filters are backwashed, the backwash water may contain high levels of (oo)cysts (Richardson et al., 1991). Ideally, backwash water should be discarded, but in many circumstances this is uneconomical. If it is recycled, treatment with coagulation and sedimentation or microfiltration will reduce recontamination of the water with (oo)cysts. Where such treatment is not feasible, it is recommended that the recycled water is returned at a constant, low rate (Rose, Lisle & LeChevallier, 1997).

Slow sand filtration will efficiently remove (oo)cysts, but efficiency is reduced at lower temperatures. No data are available for removal of oocysts in full-scale plants but a number of pilot-scale studies have been completed in which the removal efficiencies were generally good. Hall, Pressdee & Carrington (1994) achieved removal of better than 99.95%. In another study, heat-inactivated oocysts were added to surface water at a concentration of 4000/litre before filtration; no oocysts were found in the filtrate. At the end of the study, intact oocysts were found only in the upper 2.5 cm of the sand filter (Timms, Slade & Fricker, 1995).

Micro- and ultrafiltration can remove over 99.99% (Jacangelo et al., 1991; Adham, Jacangelo & Laine, 1994; Drozd & Schwartzbrod, 1997) as long as the integrity of the system is maintained.

Soil passage

Soil passage, used in bank filtration and infiltration, is probably an effective physical barrier against (oo)cysts. Its effectiveness depends on travel time and distance and on the composition of the soil (Mawdsley, Brooks & Merry, 1996).

Pretreatment reservoirs

Storage in reservoirs with a residence time of 5 months can reduce (oo)cyst concentrations by 99% (Ketelaars et al., 1995). Experimental evidence suggests that sedimentation of *Cryptosporidium* oocysts and *Giardia* cysts is unlikely to have a significant effect on their removal from a body of water unless they are attached to other particles (Medema et al., 1998b). Installation of pretreatment storage reservoirs also flattens peak contaminations (Ketelaars et al., 1995) and, because of the storage capacity, it is possible to stop the intake of surface water temporarily during high contamination events.

Disinfection

Disinfection with chlorine has always been an important means of preventing transmission of waterborne pathogens. High resistance to chlorine disinfection, especially of *Cryptosporidium* oocysts (Korich et al., 1990; Smith et al., 1990; Ransome, Whitmore & Carrington, 1993), however, makes the process ineffective for oocyst inactivation in drinking-water. Chlorine dioxide is slightly more effective, but still requires a high *CT* value (concentration (residual) of disinfectant *C*

× contact time T) of 78 mg·min/litre for 90% inactivation of oocysts (Korich et al., 1990). *Giardia* is less resistant to chlorine: 99.99% reduction can be achieved with a CT of 180–530 mg·min/litre, depending on the temperature and pH of the water (Hibler et al., 1987). At CT values of 4.7–28 mg·min/litre chlorine dioxide reduces *Giardia* by 99% (Leahy, Rubin & Sproul, 1987; Rubin, 1988).

Ozone is the most potent agent against (oo)cysts: at 20 °C, the CT for 99% inactivation of *C. parvum* oocysts is 3.5 mg·min/litre (Finch et al., 1993a) and for *G. intestinalis* cysts 0.6 mg·min/litre (Finch et al., 1993b). The effectiveness of ozone decreases at lower temperatures. Peeters et al. (1989) found that a residual ozone concentration of 0.4 mg/litre for 6 minutes was sufficient to kill 10 000 oocysts/ml, while Korich et al. (1990) demonstrated that 1 mg/litre for 10 minutes at 25 °C would result in a reduction in viability of 99%. Parker, Greaves & Smith (1993) reported that 3 mg/litre for 10 minutes killed all oocysts, and similar results were quoted by Ransome, Whitmore & Carrington (1993). Hence, the CT values required for inactivation of cysts and oocysts are high. Exposure of *Cryptosporidium* oocysts to multiple disinfectants has been shown to be more effective than was to be expected from any single disinfectant (Finch, Kathleen & Gyurek, 1994; Liyanage et al., 1997) and synergism between environmental stresses during sand filtration has also been observed (Parker, Greaves & Smith, 1993). The multiple stresses encountered by (oo)cysts in the environment and during treatment might limit the infectivity of (oo)cysts.

Conventional UV systems have a limited effect on *Cryptosporidium* and *Giardia* viability: doses of 110–120 mJ/cm² result in 99% inactivation of *C. parvum* oocysts (Ransome, Whitmore & Carrington, 1993), as assayed by in vitro viability methods, and 97% of *G. intestinalis* cysts (Rice & Hoff, 1981). In a recent animal infectivity study, Clancy et al. (1998) showed that pulsed and advanced UV are much more effective against *Cryptosporidium*; they obtained 99.98% inactivation at UV doses as low as 19 mJ/cm².

The results of laboratory disinfection experiments should be translated with caution to the full-scale treatment of environmental (oo)cysts. In surface-water treatment, (oo)cysts may be protected from the disinfectant because they are attached to colloids. On the other hand, (oo)cysts that have been exposed to environmental stressors may be more susceptible to disinfectants (Parker, Greaves & Smith, 1993). Moreover, the design and operation of full-scale treatment systems will, in general, be such that lower rates of inactivation are obtained than in the laboratory setting.

The removal of *Cryptosporidium* oocysts and *Giardia* cysts by various well designed and properly maintained and operated treatment processes is summarized in Table 6.

Little information is available regarding the ability of water-treatment processes to remove or inactivate oocysts of *Cyclospora* sp. At 8–10 µm diameter these are larger than *C. parvum* oocysts but smaller than *G. intestinalis* cysts, and it is likely that physical removal will be similar to that achieved with *Giardia* and *Cryptosporidium*. In an outbreak in Nepal, filtration and chlorination did not

Table 6. Removal of *Cryptosporidium* oocysts and *Giardia* cysts by treatment processes

Type of process	Removal efficiency (log ₁₀ -units)		Most important efficiency- determining factors
	<i>Cryptosporidium</i>	<i>Giardia</i>	
<i>Disinfection processes</i>			
Chlorine	0	0–2	Dose, contact time, installation design, disinfectant demand, temperature, pH (especially for chlorine), formation of toxic by-products, synergism of multiple disinfectants
Chloramines	0	0–2	
Chlorine dioxide	0	0–2	
Ozone	0–2	1–4	
Ultraviolet light	0–4	0–4	Dose at 254 nm, turbidity, solutes, system
<i>Filtration processes</i>			
Rapid sand	0–1	0–1	Filtration rate, recycling of backwash water
Slow sand	1.2–>3.7	1.2–>3.7	Presence of “Schmutzdecke”, filter depth
Diatomaceous earth	2–6	2–6	Filtration rate, filter depth, pore size, precoat thickness, filter integrity
Membrane	2–>4	2–>4	System integrity, membrane type
Coagulation/filtration	2–2.5	2–2.5	Coagulant dose, pH, temperature, installation
<i>Other processes</i>			
Soil passage	>2–>5	>2–>5	Soil composition, residence time, travel distance, presence of sediment
Reservoir storage	0.5–2	0.5–2	Residence time, reservoir design, addition of coagulants, temperature

affect the integrity of the oocysts (Rabold et al., 1994): although chlorine residuals remained at acceptable levels (0.3–0.8 ppm) and no coliform indicator bacteria were detected, *Cyclospora* sp. oocysts were found in the drinking-water supply. Little is known about survival of these oocysts in different environments or about treatments that will effectively inactivate them.

Risk assessment for the design of adequate treatment

One of the key issues in water treatment is to determine the level of treatment that is adequate, and this requires that the maximum acceptable concentrations in drinking-water of the pathogen(s) concerned be established. These could be derived from the maximum acceptable risk and the dose–response relationship for the parasites. An infection risk of 10^{-4} per year has been suggested as acceptable for pathogens in drinking-water (Regli et al., 1991). The maximum concentrations of viable (oo)cysts in drinking-water to meet this risk level are very

low (Rose, Haas & Regli, 1991; Rose, Lisle & LeChevallier, 1997). Since current techniques do not allow an evaluation of compliance with these concentrations to be evaluated, safeguarding of drinking-water should ideally involve a quantitative description, or assumed values of the protozoa concentrations in the source water and knowledge of the removal efficiency of the treatment steps.

Surface-water utilities, and groundwater utilities that may be contaminated by surface water or from other sources, should ensure that treatment processes adequate to achieve effective removal are in place. Additionally, the rate of protozoa removal and inactivation achieved in the treatment plant should be determined wherever possible, in order to establish whether acceptable concentrations of protozoa in drinking-water have been achieved. Effective protection of public health also requires control of recontamination in distribution systems and in households and management of backwash water and first flow water after RSF backwashing and after SSF skimming, which are critical control points.

Verification of efficiency of parasite removal

For routine monitoring, verification of treatment performance requires water-quality and process parameters. Several parameters have been suggested as surrogates for (oo)cyst removal by filtration processes: turbidity, particle counts (LeChevallier & Norton., 1992; Hall & Croll, 1997), clostridial spores (Payment & Franco, 1993; Hijnen et al, 1997) or aerobic spores (Niemiński, 1997), and particulate matter (USEPA, 1997). Although turbidity or particle counts of filtered water depend both on the levels in raw water and on filter performance, turbidity of 0.1–0.5 NTU or counts of fewer than 50/ml for particles larger than 3 µm below are indicative of good quality water. Direct and continuous information on (individual) filter performance is provided by on-line monitoring of turbidity or particle counts, making these very valuable tools for optimizing treatment efficiency for (oo)cyst removal.

Critical points in the filtration cycle are just after backwash or, in the case of slow sand filtration, scraping of the clogged top-layer from the filterbed. A slow increase in filtration rate or filtering-to-waste minimizes the risk of (oo)cyst breakthrough.

Disinfectant dose, contact time, residual disinfectant concentration at the end of the contact time, pH, and temperature are commonly used to monitor the performance of disinfection processes. The most critical conditions for disinfection processes are low temperatures and high turbidity in the water to be treated.

Conclusions and recommendations

Health risk assessment

The number and extent of outbreaks of waterborne disease in developed countries show that transmission of *Giardia* and *Cryptosporidium* by drinking-water

is a significant risk. In the case of *Cryptosporidium*, the absence of an adequate cure for immunocompromised patients increases the problem. Although the outbreaks in developed countries receive most attention, both outbreaks and low-level transmission of protozoa through drinking-water are likely in developed countries and developing countries alike (Fraser & Cooke, 1991; Isaac-Renton, Moorehead & Ross, 1996). Cysts and oocysts are regularly found in drinking-water (Isaac-Renton, Moorehead & Ross, 1996; Karanis & Seitz, 1996; Rose, Lisle & LeChevallier, 1997), although only a small proportion may be viable and infectious to man. A major drawback for the determination of the health significance of (oo)cysts in (drinking) water is that there are no detection methods that are both sensitive and specific for infectious (oo)cysts, and that provide a consistently high recovery.

Risk management

The protozoa, and to a lesser extent the viruses, have initiated a change in philosophy towards safeguarding of drinking-water from monitoring of the “end-product” drinking-water to monitoring of raw water and of treatment efficiency. The extreme resistance of some protozoa implies that a “zero risk” is no longer achievable. Measures should be designed to reduce (oo)cyst concentrations in raw water as far as possible, and treatment should preferably include filtration step(s). This implies that information on parasite concentrations in the raw water is necessary, as well as information on the removal efficiency of treatment processes. Quantitative risk assessment provides a tool for combining information on raw water quality (concentrations detected, recovery of the detection method, viability) and treatment efficiency (removal by different steps in the treatment) (Teunis et al., 1997).

The definition of maximum acceptable concentrations of pathogens in drinking-water based on a maximum acceptable (infection) risk level has become possible with data from studies in volunteers and from dose–response models (Haas, 1983; Dupont et al., 1995; Teunis et al., 1996). An annual infection risk level of 10^{-4} , as proposed by the United States Environmental Protection Agency, is currently used in Canada (Wallis et al., 1995), the Netherlands (Medema et al., 1995), and the USA (Rose, Lisle & LeChevallier, 1997) as the basis for determining the appropriate removal efficiency of surface-water treatment systems.

Current detection methods are generally sufficiently sensitive to determine the concentrations of *Cryptosporidium* and *Giardia* in surface water, but are often insufficiently sensitive for an accurate description of removal efficiency, which may require additional data from laboratory studies and (seeded) pilot plant studies. An alternative approach is to determine whether an adequate surrogate parameter can be found to describe removal efficiency for *Cryptosporidium*. (*Giardia* is easier to eliminate by both disinfection and filtration, and the description of treatment efficiency should therefore be targeted on *Cryptosporidium*.) Several parameters—aerobic spores, clostridial spores, particles, and algae—have

been evaluated on a limited scale as surrogates for protozoa removal, but a broader evaluation is necessary to determine their value.

Cryptosporidium poses a serious health risk to immunocompromised individuals, especially AIDS patients. All at-risk individuals should be made aware of the dangers and informed about means of avoiding exposure to (potentially) contaminated water. Boiling of tap water, use of mineral or bottled water, and not swimming in surface water or pools are some of the options for preventing exposure. Local considerations play a major role and public health authorities are encouraged to provide guidance on the safety of drinking-water for the immunocompromised and on appropriate means of reducing exposure (Anon, 1995; Juranek, 1995).

When an outbreak occurs, its size and source should be rapidly investigated and control measures implemented to prevent further transmission. Useful guidance on management of waterborne outbreaks can be found in the report of the UK group of experts (Badenoch, 1990), the CDC Guidance Manual (Juranek, 1995), and a workshop report (Anon, 1995).

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*Vibrio cholerae*¹

Description

Taxonomy and serological classification

Vibrio cholerae, a member of the family Vibrionaceae, is a facultatively anaerobic, Gram-negative, non-spore-forming curved rod, about 1.4–2.6 µm long, capable of respiratory and fermentative metabolism; it is well defined on the basis of biochemical tests and DNA homology studies (Baumann, Furniss & Lee, 1984). The bacterium is oxidase-positive, reduces nitrate, and is motile by means of a single, sheathed, polar flagellum. Growth of *V. cholerae* is stimulated by addition of 1% sodium chloride (NaCl). However, an important distinction from other *Vibrio* spp is the ability of *V. cholerae* to grow in nutrient broth without added NaCl.

Differences in the sugar composition of the heat-stable surface somatic “O” antigen are the basis of the serological classification of *V. cholerae* first described by Gardner & Venkatraman (1935); currently the organism is classified into 206 “O” serogroups (Shimada et al., 1994; Yamai et al., 1997). Until recently, epidemic cholera was exclusively associated with *V. cholerae* strains of the O1 serogroup. All strains that were identified as *V. cholerae* on the basis of biochemical tests but that did not agglutinate with “O” antiserum were collectively referred to as non-O1 *V. cholerae*. The non-O1 strains are occasionally isolated from cases of diarrhoea (Ramamurthy et al., 1993a) and from a variety of extraintestinal infections, from wounds, and from the ear, sputum, urine, and cerebrospinal fluid (Morris & Black, 1985). They are ubiquitous in estuarine environments, and infections due to these strains are commonly of environmental origin (Morris, 1990). The O1 serogroup exists as two biotypes, classical and El Tor; antigenic factors allow further differentiation into two major serotypes—Ogawa and Inaba. Strains of the Ogawa serotype are said to express the A and B antigens and a small amount of C antigen, whereas Inaba strains express only

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the A and C antigens. A third serotype (Hikojima) expresses all three antigens but is rare and unstable.

Between 1817 and 1961, six pandemics of cholera were recorded. The classical biotype was responsible for the fifth and sixth pandemics and is believed to have been associated with the earlier pandemics as well, although there is no hard evidence. The causative agent of the seventh and current cholera pandemic, which began in 1961, is the El Tor biotype. The classical biotype has been completely displaced worldwide, except in Bangladesh where it reappeared in epidemic proportions in 1982 (Samadi et al., 1983), remained prominent there for a few years, and now seems to have become extinct again (Siddique et al., 1991).

The simple distinction between *V. cholerae* O1 and *V. cholerae* non-O1 became obsolete in early 1993 with the first reports of a new epidemic of severe, cholera-like disease in Bangladesh (Albert et al., 1993) and India (Ramamurthy et al., 1993b). At first, the responsible organism was referred to as non-O1 *V. cholerae* because it did not agglutinate with O1 antiserum. However, further investigations revealed that the organism did not belong to any of the O serogroups previously described for *V. cholerae* but to a new serogroup, which was given the designation O139 Bengal after the area where the strains were first isolated (Shimada et al., 1993). Since recognition of the O139 serogroup, the designation non-O1 non-O139 *V. cholerae* has been used to include all the other recognized serogroups of *V. cholerae* except O1 and O139 (Nair et al., 1994a).

The emergence of *V. cholerae* O139 as the new serogroup associated with cholera, and its probable evolution as a result of horizontal gene transfer between O1 and non-O1 strains (Bik et al., 1995), has led to a heightened interest in the *V. cholerae* non-O1 non-O139 serogroups. There is evidence for horizontal transfer of O antigen among *V. cholerae* serogroups; Karaolis, Lan & Reeves (1995) reported that isolates of nearly identical *asd* gene (chromosomal housekeeping gene, which encodes aspartate semialdehyde dehydrogenase) sequences had different O antigens and that isolates with the O1 antigen did not cluster together but were found in different lineages. There has been elevated activity of the non-O1 non-O139 serogroups in the recent past, and localized outbreaks of acute diarrhoea caused by *V. cholerae* serogroups such as O10 and O12 have been reported (Dalsgaard et al., 1995; Rudra et al., 1996).

Pathogenicity for humans, and virulence factors

The major features of the pathogenesis of cholera are well established. Infection due to *V. cholerae* begins with the ingestion of contaminated water or food. After passage through the acid barrier of the stomach, the organism colonizes the epithelium of the small intestine by means of the toxin-coregulated pili (Taylor et al., 1987) and possibly other colonization factors such as the different haemagglutinins, accessory colonization factor, and core-encoded pilus, all of which are thought to play a role. Cholera enterotoxin produced by the adherent

vibrios is secreted across the bacterial outer membrane into the extracellular environment and disrupts ion transport by intestinal epithelial cells. The subsequent loss of water and electrolytes leads to the severe diarrhoea characteristic of cholera.

The existence of cholera enterotoxin (CT) was first suggested by Robert Koch in 1884 and demonstrated 75 years later by De (1959) and Dutta, Pause & Kulkarni (1959) working independently. Subsequent purification and structural analysis of the toxin showed it to consist of an A subunit and 5 smaller identical B subunits (Finkelstein & LoSpalluto, 1969). The A subunit possesses a specific enzymatic function and acts intracellularly, raising the cellular level of cAMP and thereby changing the net absorptive tendency of the small intestine to one of net secretion. The B subunit serves to bind the toxin to the eukaryotic cell receptor, ganglioside GM1. The binding of CT to epithelial cells is enhanced by neuraminidase.

Apart from the obvious significance of CT in the disease process, it is now clear that the production of CT by *V. cholerae* is important from the perspective of a serogroup acquiring the potential to cause epidemics. This has become particularly evident since the emergence of *V. cholerae* O139. A dynamic 4.5-kb core region, termed the virulence cassette (Trucksis et al., 1993), has been identified in toxigenic *V. cholerae* O1 and O139 but is not found in non-toxigenic strains. It is known to carry at least six genes, including *ctxAB* (encoding the A and B subunits of CT), *zot* (encoding zonula occludens toxin (Fasano et al., 1991)), *cep* (encoding core-encoded pilin (Pearson et al., 1993)), *ace* (encoding accessory cholera enterotoxin (Trucksis et al., 1993)), and *orfU* (encoding a product of unknown function (Trucksis et al., 1993)). In the El Tor biotype of *V. cholerae*, many strains have repetitive sequence (RS) insertion elements on both sides of the core region; these are thought to direct site-specific integration of the virulence cassette DNA into the *V. cholerae* chromosome (Mekalanos, 1985; Goldberg & Mekalanos, 1986; Pearson et al., 1993). The core region, together with the flanking RS sequences, makes up the cholera toxin genetic element CTX (Mekalanos, 1983).

Recent studies have shown that the entire CTX element constitutes the genome of a filamentous bacteriophage (CTX ϕ). The phage could be propagated in recipient *V. cholerae* strains in which the CTX ϕ genome either integrated chromosomally at a specific site, forming stable lysogens, or was maintained extrachromosomally as a replicative form of the phage DNA (Waldor & Mekalanos, 1996). Extensive characterization of the CTX ϕ genome has revealed a modular structure composed of two functionally distinct genomes, the core and RS2 regions. The core region encodes CT and the genes involved in phage morphogenesis, while the RS2 region encodes genes required for replication, integration, and regulation of CTX ϕ (Waldor et al., 1997). Generally, CTX ϕ DNA is integrated site-specifically at either one (El Tor) or two (classical) loci within the *V. cholerae* genome (Mekalanos, 1985). In El Tor strains, the prophage DNA is usually found in tandem arrays that also include a related genetic element known as RS1. The RS1 element contains the genes that enable phage DNA replication and integration, plus an additional gene (*rstC*) whose function is unknown but

that does not contain *ctxAB* or the other genes of the phage core region that are thought to produce proteins needed for virion assembly and secretion (Davis et al., 2000). CTX ϕ gains entry to the *V. cholerae* cell by way of the toxin-regulated pili—the surface organelles required for intestinal colonization. Its genes are then incorporated into host chromosome, inducing the cell to secrete CT.

The *zot* gene increases the permeability of the small intestinal mucosa by an effect on the structure of the intestinal tight junctions (Fasano et al., 1991), while *ace* affects ion transport in the intestinal epithelium. Another factor whose gene resides outside the CTX genetic element and which is thought to contribute to the disease process is haemolysin/cytolysin (Honda & Finkelstein, 1979). In contrast to the watery fluid produced by CT, the haemolysin can cause accumulation in ligated rabbit ileal loops of fluid that is bloody with mucous (Ichinose et al., 1987). Although not fully characterized, other toxins produced by *V. cholerae* include the shiga-like toxin (O'Brien et al., 1984), a heat-stable enterotoxin (Takeda et al., 1991), new cholera toxin (Sanyal et al., 1983), sodium channel inhibitor (Tamplin et al., 1987), thermostable direct haemolysin-like toxin (Nishibuchi et al., 1992), and a cell-rounding cytotoxic enterotoxin known as the non-membrane-damaging cytotoxin (Saha, Koley & Nair, 1996; Saha & Nair, 1997).

In vitro and animal studies, volunteer studies, dose–response modelling

Although natural infection with *V. cholerae* O1 does not occur in animals, some animal models have been developed for the study of cholera. A few of these models have yielded useful information relevant to human disease. The most widely used adult intact animal model for *V. cholerae* is the RITARD (removable intestinal tie-adult rabbit diarrhoea) model (Spira, Sack & Fröhlich, 1981), which allows massive and often fatal diarrhoea to occur within 1–5 days.

Volunteer studies with *V. cholerae* have yielded many insights into pathogenesis and host immune response. It has been shown (Cash et al., 1974; Levine et al., 1988) that more than 10^8 *V. cholerae* cells are required to induce infection and diarrhoea. Administration of sodium bicarbonate (NaHCO_3) to neutralize gastric acid dramatically reduces the infectious dose to less than 10^4 , although lower inocula correlate with longer incubation periods and decreased stool volumes.

Disease occurrence, outbreaks, sporadic cases, prospective studies

Cholera has re-emerged as a major infectious disease in the recent past, with a global increase in its incidence. In 1994 cholera cases were notified from 94 countries—the highest ever number of countries in one year (World Health Organization, 1995). Two particularly disturbing aspects of the global cholera picture in the 1990s have been the dramatic and unexpected reappearance in

January 1991 of epidemic cholera caused by *V. cholerae* O1 El Tor in Latin America after a 100-year absence from the region (Tauxe & Blake, 1992) and the unprecedented appearance in late 1992 in southern India of an epidemic strain of *V. cholerae* non-O1, classified as *V. cholerae* O139 Bengal (Ramamurthy et al., 1993b). The reasons for these phenomena are still being intensively researched.

Asia

A new cholera epidemic erupted in Madras, southern India, in October 1992 and rapidly spread eastward (Ramamurthy et al., 1993b). The causative organism isolated from this outbreak was *V. cholerae* non-O1, which produces cholera toxin and is now known as the O139 serogroup. Within months of the Madras outbreak, *V. cholerae* O139 strains were isolated from Calcutta and Bangladesh (Albert et al., 1993; Ramamurthy et al., 1993b). The O139 serogroup has spread rapidly into several countries in south-east Asia since 1992, raising the concern that this may be the beginning of the eighth pandemic (Bhattacharya et al., 1993; Nair et al., 1994b). In 1994, however, a dramatic decline in the incidence of cholera caused by serogroup O139 was observed in areas where it had predominated in the preceding years. The O139 was replaced by strains of the O1 serogroup (Mukhopadhyay et al., 1996), but genetic studies showed them to be different from the O1 strains that were circulating before the emergence of the O139 serogroup (Faruque et al., 1997; Sharma et al., 1997; Yamasaki et al., 1997). In August 1996 there was a resurgence of the O139 serogroup—with an altered antibiogram—in Calcutta and other parts of India (Mitra et al., 1996), replacing the existing O1 serogroup to become the dominant serogroup in this part of the subcontinent. Molecular studies have again demonstrated that the O139 strains that re-emerged in August 1996 showed changes at the genetic level and were different from the O139 strains that appeared in 1992 (Sharma et al., 1997). A total of 50 921 cases and 1145 deaths were reported from 18 countries in Asia in 1995. The number of countries reporting thus declined from 26 in 1994. The case-fatality rate increased from 1.3% in 1994 to 2.2% in 1995 (World Health Organization, 1996).

Several lines of evidence suggest that the O139 serogroup closely resembles the O1 El Tor biotype. However, O139 contains a distinct O antigen and has been shown to express a polysaccharide capsule (Johnson et al., 1994; Weintraub et al., 1994). Accumulating data suggest that serogroup O139 Bengal arose from a serogroup O1 biotype El Tor by deletion of the genes responsible for O1 antigen biosynthesis (Manning, Stroehrer & Morona, 1994; Waldor & Mekalanos, 1994; Comstock et al., 1995) and acquired DNA from another non-pathogenic serogroup (Mooi & Bik, 1997). In a cholera-endemic area, a newly emergent non-O1 serogroup has a selective advantage because of the absence of pre-existing immunity. These observations indicate the ability of *V. cholerae* to react to change and to adverse conditions (such as immunity).

Africa

From 1970, *V. cholerae* O1 El Tor has gradually spread to most of the continent with case-fatality rates between 4% and 12%. From 1991 to 1996, the number of cases remained high and ranged between 70 000 and 160 000 (World Health Organization, 1997). The largest proportion of all reported cholera cases in 1994, and 42% of all cholera deaths reported globally that year, were in Africa (World Health Organization, 1995). The impact of war and political unrest on diarrhoeal disease is illustrated clearly by the cholera epidemic in Rwanda, which can be categorized into two phases. During the “stay” phase, nearly 2 million people resident in Rwanda moved to neighbouring countries because of the 1994 civil war between Hutu and Tutsi tribes. About 1 million Rwandans fled to Goma, Zaire (now the Democratic Republic of the Congo), and stayed in makeshift camps. Among these refugees, 12 000 died during epidemic outbreaks of cholera and shigellosis, caused mainly by poor water-supply and sanitation facilities coupled with inadequate use of oral rehydration therapy, use of inappropriate intravenous fluids, and inadequate experience among health workers (Siddique et al., 1995). Surveys conducted in the Goma region showed that the epidemic was caused by multidrug-resistant *V. cholerae* O1 biotype El Tor and *Shigella dysenteriae* type I (Islam et al., 1995). In the “return” phase in 1996, 8916 cases of diarrhoea were recorded among the 350 000 Rwandans returning from five camps. The very low case-fatality rates were attributed to the rapid response by health officials in the cholera treatment centres (Brown et al., 1997). In 1995, a decrease of about 44% in the number of cases was observed compared with 1994. Efforts to repatriate refugees, improvements in surveillance, and control of diarrhoeal diseases by governments and collaborating agencies contributed to the decline in incidence and case-fatality rates. As of December 1996, 26 countries had reported cholera, with Nigeria, Senegal, and Somalia reporting more than 1000 cases each and very high case-fatality rates (World Health Organization, 1997).

Latin America

In 1991 cholera appeared in Latin America—the last part of the less-developed world to have remained untouched by the seventh pandemic of cholera. The epidemic in Peru began in three different foci along the Pacific coast (Ries et al., 1992). The Pan American Health Organization reported that 750 000 cases of cholera with 6500 deaths occurred between 1991 and 1992 (Tauxe et al., 1994). The epidemic spread in conventional fashion, following the trade routes into the interior of Peru, Ecuador, Colombia, Brazil, Chile, and then central Mexico. One year after the appearance of cholera in Peru in 1991, 18 south and central American countries had reported cases of cholera.

The appearance of cholera in Latin America remains an enigma. It could have been introduced by maritime traffic from the Pacific region, just as the Latin

American epidemic strain was introduced into the USA's Mexican Gulf coast in 1991 (McCarthy et al., 1992). Coincidental to cholera in Peru was a warm event related to El Niño in the tropical Pacific from 1990 to 1995 (Colwell, 1996). The Latin American isolates of *V. cholerae* were different from the endemic strain in the USA but share several similarities with most of the seventh pandemic isolates (Wachsmuth, Bopp & Fields, 1991; Faruque & Albert, 1992). All the Latin American strains subjected to multilocus enzyme electrophoresis exhibited the same pattern (Wachsmuth et al., 1993), indicating that they are clonal. However, when the Latin American clone of *V. cholerae* O1 was compared with three other known global clones—the seventh pandemic clone, the Mexican Gulf coast clone, and the Australian clone—the multilocus enzyme electrophoresis pattern of the Latin American clone was distinct from that of previously known clones of *V. cholerae* O1. It has been suggested that *V. cholerae* O1 was introduced into the aquatic environment off the Peruvian coast long before the outbreak flourished.

The Peruvian epidemic illustrates the importance of preparing for cholera epidemics. As many as 4500 cases a day occurred during the Peruvian epidemic, yet the *mortality rate* was less than 1% (Anon, 1991).

International travel and cholera

During the nineteenth century cholera was a classic disease of long-distance travellers. More recently, international travel has often been the cause of sporadic cases of cholera in Chile and the USA. A history of seafood consumption is often obtained from travellers who acquire cholera during visits to areas in which the disease is endemic. Rarely, *V. cholerae* non-O1 non-O139 has been implicated as the cause of acute gastroenteritis among travellers with diarrhoea (Bhattacharya et al., 1992).

Some 38 cholera cases associated with consumption of shellfish from coastal waters in the Gulf of Mexico were reported in the USA in the 15 years from 1973 (Popovic et al., 1993). Shellfish was the vehicle of transmission of cholera from Latin America to the USA on five separate occasions in 1991. Isolation of toxigenic *V. cholerae* strains from commercial oyster beds in Mobile Bay, Alabama, raised concerns about contamination of shellfish beds in the Gulf of Mexico with Latin American *V. cholerae* strains (Depaola et al., 1992). However, molecular typing methods clearly showed that the Latin American isolates were different from previously described Gulf coast strains (Wachsmuth, Bopp & Fields, 1991). Furthermore, samples of the ballast, bilge, and sewage from several ships arriving from Latin American ports and docking in Gulf of Mexico ports has revealed the same toxigenic strains of *V. cholerae* O1 as those found in the contaminated oyster beds in the Gulf of Mexico (McCarthy et al., 1992). These data support the hypothesis that ships were responsible for the initial introduction of *V. cholerae* O1 to coastal waters of the Gulf of Mexico.

Monitoring and assessment

Sampling and sample preparation

For the investigation of surface waters, water samples should be collected in sterilized bottles following standard procedures. Plants should be collected in sterile polyethylene bags, and phytoplankton and zooplankton should be collected using plankton nets and kept in sterile glass bottles. Sediment should be collected by a core sampler and kept in sterile polyethylene bags. All field samples should be transported to the laboratory inside a cooled container (at about 4–10°C) and processed within 6 hours (Donovan & van Netten, 1995).

Analytical methods: culture methods, immunological and molecular methods, methods performance

A qualitative enrichment procedure is normally performed for the detection of *V. cholerae* from food or environmental samples. Quantitative procedures, either direct plating or most probable number (MPN), are required only occasionally. Culture media that were developed for the isolation of *V. cholerae* from faeces in clinical laboratories have also generally been used for the isolation of *V. cholerae* from foods or the environment. Alkaline peptone water (APW) is the standard medium for enrichment of *V. cholerae*, although several nutrient-rich modifications of APW, such as blood–APW and egg–APW are also used (Donovan & van Netten, 1995). Thiosulfate–citrate–bile-salts–sucrose agar (TCBS) is a highly selective differential medium that is most commonly used for the isolation of *V. cholerae*; its selective ingredients suppress the growth of most of the interfering organisms such as coliforms, pseudomonads, aeromonads, and other Gram-positive bacteria (Kobayashi et al., 1963). The advantage of TCBS is its sucrose–bromthymol blue diagnostic system, which distinguishes the yellow sucrose-positive colonies of *V. cholerae* from other colonies.

For isolation of *V. cholerae* from the environment the following procedures are recommended:

- 10 g of plant material are homogenized with 100 ml of normal saline in a blender.
- 10 ml of plankton sample should be homogenized in a PTFE-tipped tissue grinder using a stirrer.
- 1 ml of plant homogenate, 10 ml of plankton homogenate, 50 ml of water, and 1.0 g of sediment are enriched in either APW or bile–peptone broth overnight at 37°C (Islam, Alam & Khan, 1995).
- All samples are then plated on TCBS agar or taurocholate–tellurite–gelatin agar and incubated at 37°C for 18–24 hours.

Suspected *V. cholerae* strains transferred from primary isolation media can be identified by means of a standard series of biochemical media used for identifi-

cation of members of the Enterobacteriaceae and Vibrionaceae families. Both conventional tube tests and commercially available enteric identification tests are suitable for identifying *V. cholerae*. A crucial test for differentiation of *V. cholerae* from Enterobacteriaceae is the positive oxidase test. Other key traits for distinguishing *V. cholerae* from other species include fermentation of D-glucose with acid production (without gas), maltose, D-mannitol, sucrose, and trehalose. Most strains are also motile at 37 °C, metabolize lysine and ornithine, and show a positive string test (a mucoid “string” is formed when a large loop of growth from a noninhibitory agar medium is suspended in a drop of 0.5% aqueous selection deoxycholate and then drawn). The absence of arginine metabolism is also frequently used for differentiation. However, the most important test for identification of *V. cholerae* O1 or O139 is agglutination in antisera raised against O1 or O139.

Various simpler schemes for identification of *V. cholerae* are available for use in developing countries. One involves the inoculation of suspected *V. cholerae* colonies from the isolation plate into a single-tube, multitest medium which is based on the principles of triple sugar iron (TSI) and Kligler iron agar (KIA) medium (Kaper, 1979). Cultures yielding an alkaline slant (K) over acid (A) butt, with no gas or H₂S, are then tested for oxidase and reactivity with O1 or O139 antisera, using growth taken from the multitest medium. Extensive evaluation has revealed that 97.9% of the oxidase-positive strains that yield a K/A reaction in the multitest medium have biochemical reactions consistent with those of *V. cholerae* (Nair et al., 1987). Strains of *V. cholerae* that do not agglutinate in either O1 or O139 antisera should be labelled as non-O1 non-O139; if further serogrouping is deemed necessary it should be done at an International Reference Center for serogrouping of *V. cholerae*.

Specific probes for the A and B subunit genes of CT have been used to detect the location of these genes in the *V. cholerae* genome and in differentiating between clones of *V. cholerae* (Kaper et al., 1982; Wachsmuth, Bopp & Fields, 1991; Wright et al., 1992). Restriction fragment length polymorphism of the enterotoxin gene has been successfully used to identify the origin of *V. cholerae* strains involved in outbreaks (Yam et al., 1991). The DNA sequences of the structural genes of the CT subunit B show heterogeneity and have been classified into three genotypes: genotype 1 is found in strains of classical biotype worldwide and El Tor biotype strains associated with the USA Gulf of Mexico coast, genotype 2 in El Tor strains from Australia, and genotype 3 in El Tor biotype strains from the seventh pandemic and the recent Latin American epidemic (Ølsvik et al., 1993). Molecular diagnostic tests, such as PCR, are now being developed for both clinical and environmental monitoring of *V. cholerae* O1 and O139. Primer pairs corresponding to unique stretches in the genes of the *rfb* complex, which encode the O antigen, have been designed to develop PCRs for specific detection of O1 (Hoshino et al., 1998) and O139 (Albert et al., 1997) from stool specimens.

Control

Emission, transport, and survival in the environment

Most *Vibrio* species are ubiquitous in estuarine and marine environments and are also found in fresh water provided that there is a certain minimal level of sodium ions. The cholera vibrio, however, was long considered to be an exception, in that it was believed not to be an environmental organism (Feachem, Miller & Drasar, 1981) but associated with water only as a result of sewage contamination. Thus, until the late 1970s, *V. cholerae* was considered by most workers in the field to be an organism whose normal habitat was the human gut and to be incapable of surviving for more than a few days outside the gut. The reason for this belief was a general failure to isolate the organism from the water unless there were cholera cases in the immediate vicinity. During epidemics, toxigenic *V. cholerae* O1 or O139 can be isolated from the local fresh water as well as from patients (Khan et al., 1984; Ghosh et al., 1994) but disappears from the environment after the epidemic subsides.

Since the early epidemiological work in 1853 in London by John Snow and later laboratory investigation by Robert Koch (1884), it has been known that water is important in the transmission of cholera. Water from public supplies was implicated in the previous six pandemics. In the present (seventh) pandemic, properly treated public water supplies are not generally considered to be a risk factor. However, serious epidemics of cholera continue to occur in areas where treatment is poor or sporadic, such as in China and the Russian Federation, in Latin America, and in other developing countries. The recent epidemic in Latin America was said to have been exacerbated by the failure of the authorities to chlorinate water supplies; this omission was due in part to concern about the carcinogenic effect of chlorination by-products in drinking-water.

While there is no doubt that the faecal–oral route of cholera transmission is of primary concern because of its importance in the development of secondary cases and in the subsequent spread of the disease, it does not fully explain seasonal recurrence of the disease in some areas or outbreaks that occur where faecal–oral transmission is unlikely. Traditional culture techniques for isolating *V. cholerae* from water are frequently unsuccessful. More advanced techniques, however, using direct immunofluorescence microscopy, DNA hybridization, PCR, and improved culture methods, have frequently isolated both O1 and non-O1 strains, even in the absence of traditional faecal indicator bacteria such as *Escherichia coli* and faecal streptococci. This suggests either that *V. cholerae* can survive longer in the environment than other faecal organisms or that *V. cholerae* is an environmental organism in its own right.

Several surveys have been conducted to study the environmental distribution of *V. cholerae* in diverse areas in the world. From their results it is clear that *V. cholerae* is widely distributed in temperate and tropical aquatic environments. Its distribution is affected by various abiotic factors including the inorganic and organic contents of water and sediments, pH, fluctuating temperature, salinity,

variations in oxygen tension, and exposure to the ultraviolet rays in sunlight. A linear correlation with salinity was observed, with more frequent isolations at sites with salinities between 0.2‰ and 2.0‰. The effect of temperature was more strongly correlated with the frequency of isolations when the water temperature was above 17°C. Studying the influence of water temperature, salinity, and pH on survival and growth of toxigenic *V. cholerae* O1 associated with live copepods, Huq et al. (1984) concluded that 15‰ salinity, 30°C water temperature, and pH 8.5 supported increased attachment and multiplication of *V. cholerae* on copepods.

The cholera vibrio has been found in association with a wide range of aquatic life, including cyanobacteria (*Anabaena variabilis*) (Islam, Drasar & Bradley, 1989), diatoms (*Skeletonema costatum*) (Martin & Bianchi, 1980), in freshwater filamentous green algae (*Rhizoclonium fontanum*) (Islam, Drasar & Bradley, 1989), oysters (*Crassostrea virginica*) (Hood, Ness & Rodrick, 1981), water hyacinths (*Eichornia crassipes*) (Spira et al., 1981), the arthropod *Gerris spinolae* (Shukla, Singh & Sanyal, 1995), and blue crab (*Callinectes sapidus*) (Huq et al., 1986). *V. cholerae* produces a chitinase and is able to bind to chitin, a semi-transparent material, predominantly mucopolysaccharide, that is the principal component of crustacean shells (Nalin et al., 1979; Colwell & Spira, 1992). It colonizes the surfaces of copepods (Huq et al., 1983; Tamplin et al., 1990) with preferential attachment to the oral region and the egg sac. *V. cholerae* O1 also attaches to *Volvox* sp., a colonial form of phytoplankton, and the attachment appears as a “ring” pattern (Colwell et al., 1990).

Chitinases and mucinases facilitate the attachment of *V. cholerae* to aquatic organisms, while algae surface films enhance the growth of the pathogen (Epstein, Ford & Colwell, 1993). It has been suggested that *V. cholerae* can survive an inter-epidemic period and colonize the surfaces of algae, phytoplankton, and water hyacinth (Islam, Alam & Neogi, 1992). In an epidemic area like the Ganges river delta, copepods favour survival of *V. cholerae* because of the organism's production of chitinase and ability to use chitin as a source of nutrients (Nalin, 1976).

When present in the environment, *V. cholerae* may undergo a series of major physical and metabolic changes; while the altered cells are more difficult, or even impossible, to grow using conventional techniques, they have been shown to retain their virulence factors. Colwell et al. (1984) suggest that *V. cholerae* O1 can assume, or approximate, a state of dormancy in response to nutrient deprivation, elevated salinity, and/or reduced temperature. This finding was supported by others, who reported strong linear correlations between *V. cholerae* non-O1 and temperature and salinity (Miller, Drasar & Feachem, 1984). Nutritionally deprived *V. cholerae* shows an initial rapid decline in total lipids and carbohydrates and a more gradual decline in proteins and DNA (Hood, Guckert & White, 1986).

The O1 and O139 strains may produce CT and other virulence factors; non-O1 non-O139 strains rarely possess these attributes. Laboratory studies have shown no loss of toxigenicity by *V. cholerae* O1 under conditions of low salinity, adverse pH, adverse water chemistry, low sodium, or long-term starvation, sug-

gesting that toxin-producing ability is unlikely to be lost when the organism is exposed to environmental stress (Miller et al., 1986). Survival of toxigenic *V. cholerae* O1 in water at different temperatures and with different values of salinity, pH, and cation concentration and composition also indicate its ability to survive for extended periods in warm water (25 °C) containing no nutrients, with a salinity of 0.25–3.0‰ and a pH of around 8.0 (Miller, Drasar & Feachem, 1984). Some strains have shown increased toxin production under certain conditions, such as when attached to various aquatic plants (Islam, 1990). The numbers of *V. cholerae* suspended in water are generally low, approximately 10³ cfu/litre for non-O1s and less than 50 cfu/litre for O1s. However, the organism can multiply rapidly in badly stored drinking-water and may be found in large numbers associated with aquatic species such as cyanobacteria, algae, zooplankton, and crustacea (including commercial species such as crabs). While counts of free organisms in the water may be low, copepods found in the same water may have 10⁵ organisms attached to their surface (Huq et al., 1983).

The continuing failure of attempts to isolate toxigenic *V. cholerae* O1 from natural aquatic environs remains unexplained. Although toxigenic *V. cholerae* has been isolated from surface waters, no study has yet demonstrated water as a reservoir of toxigenic *V. cholerae* in the absence of a person with cholera using that water. Organisms of the O1 serogroup have frequently been isolated from aquatic environs, but most of the environmental O1 isolates do not produce CT, the toxin to which the clinical state of cholera is principally attributed. Even in a hyperendemic area like Calcutta, toxigenic *V. cholerae* O1 could not be isolated from several aquatic bodies examined for a year (Nair et al., 1988).

The overall body of evidence suggests that faecal–oral transmission is of primary importance, and long experience has shown basic water-supply and sanitation measures to be effective in controlling secondary spread of the disease. Such measures are essential since toxigenic *V. cholerae* may be reintroduced to nonendemic areas by several mechanisms. It is also likely that environmental survival during inter-epidemic periods accounts for sudden multi-point outbreaks of cholera as occur, for example, in the Ganges delta area.

Effects of drinking-water treatment

Ensuring safe drinking-water implies both securing a safe source and maintaining safety up to the point of consumption. This is equally true of sophisticated piped distribution systems, of water collected by householders from sources such as wells, and of water provided to the consumer by any other means.

Groundwater sources such as wells and springs are often believed to be of good quality with regard to bacterial pathogens transmitted by the faecal–oral route. However, such sources are readily contaminated by faecal material, especially where there are potential sources of contamination nearby or where contaminants may be carried by surface waters. Protection measures need to be properly applied (see for example World Health Organization, 1997).

Surface-water sources should generally be considered to be susceptible to faecal contamination and, therefore, to contamination by *V. cholerae*. However, the organism can be easily eliminated from drinking-water by appropriate treatment.

It has long been accepted that slow sand filtration is effective for removal of *V. cholerae* during drinking-water treatment. The biological processes that are responsible for water purification occur more slowly at low temperatures, and ice formation on filter surfaces has been associated with unacceptable deterioration in effluent water quality. The use of open filters should therefore be avoided in regions where temperatures can drop below 0°C.

Other common treatment methods such as coagulation, flocculation, sedimentation, and rapid filtration will significantly reduce numbers of *V. cholerae*, but should be seen as preparatory treatments to be followed by disinfection.

Most chemical disinfectants effectively eliminate *V. cholerae* under normal operating conditions (principally concentration and time) provided that water is clear (i.e. free of particulates). The same is true of physical disinfection methods, such as the use of ultraviolet light.

Recontamination of "safe" water is a significant concern. When water has to be collected, there are several opportunities for recontamination, by recipients, and during handling or extraction from storage for use. In piped supply systems recontamination is also a significant risk, especially where the supply is discontinuous or of low pressure and where there is appreciable leakage. To minimize the health risks associated with recontamination, use of a residual disinfectant is recommended.

Boiling is generally advised but for poor populations this is not affordable (Barua & Merson, 1992). Use of potassium aluminium sulfate ($K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 24H_2O$), 500 mg/litre, has been claimed to kill *V. cholerae* (Barua & Merson, 1992) but the taste of water is unacceptable to many. Chlorine-releasing agents (such as calcium hypochlorite or bleaching powder) are very effective and less expensive. For domestic chlorination 1% stock solution is prepared by adding enough water to 4 teaspoons (16 g) of hypochlorite or 10 teaspoons (40 g) of bleaching powder to make 1 litre. Three drops of stock solution should be added per litre of water, which should be allowed to stand for 20–30 minutes before use (Clark, 1956). Various disinfecting solutions, containing about 1% chlorine, are available commercially, as are water purification tablets and liquid preparations containing chlorine. It is important that the stability of the disinfectant is checked frequently and that the disinfected water is properly stored. Iodine is also an excellent disinfectant for water: two drops of 2% tincture of iodine are sufficient for 1 litre of water. Various iodine-containing preparations are commercially available.

Water from a relatively protected source cannot be guaranteed to be free from contaminating bacteria when actually consumed. In a typical urban setting in a developing country, where the water supply is intermittent, drinking-water is collected and stored, in various ways, for one or more days in the household. If the water is not handled correctly, the processes of collection and storage provide

ample opportunity for contamination. In addition, the residual chlorine in stored water is relatively low, and the viability of *V. cholerae* is thus extended. It has been recently reported that *V. cholerae* O1 and non-O1 can shift to a rugose form associated with the production of an exopolysaccharide which promotes cell aggregation (Morris et al., 1993). This rugose form resists both chlorine (even at levels exceeding 2 mg/litre) and other disinfectants in potable water and is likely to contribute to the waterborne transmission of cholera (Rice et al., 1992; Morris et al., 1993). Contamination of drinking-water can also occur as result of wastewater influx in old or damaged network systems. Network maintenance must therefore be considered as a further preventive measure against cholera.

Little is known about the regrowth ability for *V. cholerae* as a result of the uptake of assimilable organic carbon (AOC) or about the organism's occurrence or colonization in biofilms. In Africa, however, the short duration of the cholera epidemics in Goma and Uvira, which are connected by Lake Kiva, Lake Tanganyika, and the Rusizi river, suggest that large freshwater bodies do not provide a suitable environment for *V. cholerae* (Birmingham et al., 1997). This might be because of the low concentration of AOC (West, 1989). Cholera outbreaks in Burundi, including that in Rumonge, associated with natural water sources, were reportedly due to faecal contamination and high levels of AOC (Birmingham et al., 1997).

Conclusions and recommendations

Cholera is usually transmitted by the faecal-oral route, with the infecting dose being around 10^8 . Individuals with reduced gastric acidity and blood group O are more susceptible to infection. In situations where poor environmental sanitation is coupled with poor domestic and personal hygiene, transmission of cholera is a result of faecal contamination of drinking-water. Events such as flood, famine and war, resulting in movements of refugees, favour the outbreak and spread of cholera. Refugees may be at particular risk for cholera because of secondary contamination, overcrowding, inadequate sanitary facilities and water supplies, and malnutrition. The situation is exacerbated by a number of other problems, such as inadequate staffing, frequent shortages of oral rehydration salts and late presentation of cases because of transport difficulties.

A study in Calcutta has shown that, at the family level, faecal contamination of stored water can be prevented by the use of narrow-necked pitchers.

It has been suggested that the control of cholera epidemics is too big a task for a national diarrhoeal diseases control programme. Although this may be true in the case of extensive epidemics, it should not be forgotten that large outbreaks of this kind are often the result of initial delays in detection and containment. A properly organized national control programme provides the framework for quick detection and prompt containment and is the best means of ensuring preparedness for cholera control. Some strengthening of existing human and material resources may be required, but this is much easier than ad hoc efforts to fight a large and widespread epidemic.

The essential features of a national diarrhoeal diseases control programme include a national epidemic control committee, a well-established surveillance system, environmental sanitation and safe water supplies, health education, and hands-on training in clinical management (with adequate laboratory and logistic support). During an epidemic, the critical elements of cholera control are early identification of cases through surveillance and case-finding, notification to health authorities and WHO, establishment of treatment centres, health education, and proper disposal of human waste. Except in special circumstances, there is little value in chemoprophylaxis or mass vaccination. Restrictions on trade and travel offer few advantages, but travellers to epidemic areas should be extremely careful about what they eat and drink and scrupulous about personal hygiene.

Under the International Health Regulations, notification of WHO about cases of cholera is mandatory. National health authorities should report the first suspected cases to WHO at the earliest possible moment; laboratory confirmation of cases should also be reported immediately. Thereafter, health authorities should report confirmed cases to WHO on a weekly basis. Once the presence of cholera in a particular area has been confirmed, it is unnecessary to confirm all subsequent cases. Moreover, neither the treatment nor the notification of suspected cases of cholera requires laboratory confirmation of the presence of *V. cholerae* O1 (World Health Organization, 1993): the decision to initiate anti-epidemic measures must be taken regardless of the causative strain.

National risk management strategies should be planned and implemented by a national coordinating committee. This committee should be responsible for cholera preparedness, intersectoral cooperation, regional and interregional collaboration, collection and reporting of information on cholera cases and deaths, organization of any special training that may be required, procurement, storage and distribution of essential supplies, and implementation, supervision, monitoring and evaluation of control activities (World Health Organization, 1993).

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This Addendum to the second edition of the *Guidelines for drinking-water quality* contains reviews of a number of pathogens that occur widely in water – *Aeromonas* and *Legionella* spp., *Vibrio cholerae*, enteric hepatitis viruses, and protozoan parasites (*Cryptosporidium*, *Giardia*, and *Cyclospora* spp.). These review documents supersede, and are more comprehensive than, those previously published in Volumes 1 and 2 of the *Guidelines*.

Unlike the analogous chemical reviews in the *Guidelines*, the microbiological review documents do not conclude with “safe” or “tolerable” exposure levels for the pathogens. The microbiological quality of drinking-water can vary rapidly and widely, and even brief exposures to pathogens may have serious health consequences. Analytical techniques for recognized pathogens may be time-consuming and complex, and are not always available, and knowledge of the identity of waterborne pathogens is in any case incomplete. For these reasons, defining safe exposure and monitoring its achievement are not generally the preferred means of control; a more effective approach lies in understanding the conditions that are likely to ensure the safety of drinking-water supplies, and in more direct monitoring of the fulfilment of these conditions. The microbiological reviews therefore summarize current knowledge of transmission, attenuation, and removal of the individual pathogens, and of the effectiveness of measures for interrupting transmission.