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Diversity, Sources, and Detection of Human Bacterial Pathogens in the Marine Environment

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2.1. INTRODUCTION

Disease outbreaks in marine organisms appear to be escalating worldwide (Harvell et al., 1999, 2002) and a growing number of human bacterial infections have been associated with recreational and commercial uses of marine resources (Tamplin, 2001). Whether these increases reflect better reporting or global trends is a subject of active research (reviewed in Harvell et al., 1999, 2002; Rose et al., 2001; Lipp et al., 2002); however, in light of heightened human dependence on marine environments for fisheries, aquaculture, waste disposal, and recreation, the potential for pathogen emergence from ocean ecosystems requires investigation.

A surprising number of pathogens have been reported from marine environments and the probability of their transmission to humans is correlated to factors that affect their distribution. Both indigenous and introduced pathogens can be the cause of illness acquired from marine environments and their occurrence depends on their ecology, source, and survival. To judge the risk from introduced pathogens, levels of indicator organisms are routinely monitored at coastal sites. However, methods targeting specific pathogens are increasingly used and are the only way to judge or predict risk associated with the occurrence of indigenous pathogen populations.

In this chapter, we review the recognized human pathogens that have been found in associations with marine environments (Section 2.2), the potential routes of transmission of marine pathogens to humans, including seafood consumption, seawater exposure (including marine aerosols), and marine zoonoses (Section 2.3), and we discuss the methods available to assess the public-health risks associated with marine pathogens (Sections 2.4 and 2.5).

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2.2. DIVERSITY AND ECOLOGY

Our current knowledge of the diversity and ecology of bacterial pathogens associated with marine environments stems from (i) clinical accounts of marine-acquired illnesses, (ii) disease outbreaks of known etiology in marine animals, and (iii) testing of marine environments for the presence of pathogen populations. In particular, surveys of environmental microbial communities based on 16S ribosomal RNA (rRNA) gene sequence diversity have revealed a large number of organisms closely related to human pathogens; however, the public health risk of many of these pathogen-like populations remains unknown. This is largely due to a poorly defined relationship between clinical isolates and pathogen-like populations detected in the environment because many methods used to detect environment populations do not possess high enough resolution to discriminate virulent from harmless strains.

The genetic elements encoding virulence properties are not uniformly distributed among strains within a potentially pathogenic species. For marine pathogens, this has been explored in some detail in *Vibrio* species. Environmental populations of *Vibrio* are characterized by heterogeneous distributions of multiple virulence factors, combinations of which regulate the epidemic potential (e.g. Faruque et al., 1998; Karaolis et al., 1998; Chakraborty et al., 2000). Similarly, comparisons of the genomic diversity of clinical and environmental *Vibrio vulnificus* isolates suggest that seafood-borne human infections are established by a single highly virulent strain among coexisting genetically heterogeneous populations (Jackson et al., 1997). However, what leads to the occurrence of one strain over another remains poorly understood.

Whether environmental conditions select for strains possessing human virulence factors is an area of increased research (e.g. Tamplin et al., 1996; Jackson et al., 1997; Faruque et al., 1998; Chakraborty et al., 2000). Such factors may include attachment mechanisms to organic matter, motility, secretion of lytic compounds, and the ability to grow rapidly under nutrient-replete conditions. Transfer of virulence properties between different species has been observed (Faruque et al., 1999; Boyd et al., 2000), and specific virulence factors (e.g., hemolysins, toxins, attachment pilli) may be borne on mobile genetic elements. Thus, environmental interaction may confer enhanced pathogenicity on a subset of an environmental population. In general, the marine environment may be a powerful incubator for new combinations of virulence properties due to the extremely large overall population size of bacterial populations and efficient mixing timescales. These natural phenomena may be further enhanced by human activity such as increased sewage input and ballast water transport (Ruiz et al., 2000) both of which introduce microbial species across geographical barriers.

2.2.1. Pathogenic Species

The known diversity of human pathogens in the ocean continues to expand as the virulence of emerging pathogens is recognized. Pathogens associated with marine environments and their observed routes of transmission to humans are presented in Table 2.1. Of the 23 lineages currently characterized within the domain Bacteria by 16S rRNA phylogeny (Cole et al., 2003), six harbor human pathogens, and of these six, all lineages contain strains found as human and/or animal pathogens in marine environments (i.e., the Bacteroides-Flavobacterium group (Bernardet, 1998), the Spirochetes, the Gram-positive Bacteria, the Chlamydia (Johnson & Lepennec, 1995; Kent et al., 1998), the Cyanobacteria (Carmichael, 2001), and the Proteobacteria) (see also references in Table 2.1).

Table 2.1. Human-pathogenic bacteria detected in marine environments.

Genus	Species[a]	Hosts of marine disease: Humans	Hosts of marine disease: Marine animals	Observed routes of human infection[b]: Seafood	Sea water	Zoonoses	Aerosols	Estimated infectious dose[c]	Human syndrome	References
Acinetobacter	*A. calcoaceticus*	ND	X				X		Sepsis, meningitis, pneumonia (nonmarine acquired)	(Austin et al., 1979; Grimes et al., 1984; Grimes, 1991)
Aeromonas	*A. hydrophila*	X		X	X			10^7 and 10^9 cells in 2 of 57 tests (Morgan, 1985)	GI, sepsis, wound infection	(Morgan et al., 1985; Chowdhury et al., 1990; Ashbolt et al., 1995; Jones & Wilcox, 1995; Caudell & Kuhn, 1997; Fiorentini et al., 1998; Dumontet et al., 2000)
	A. caviae	X		X	X				Wound infection	(Ashbolt et al., 1995; Jones & Wilcox, 1995; Dumontet et al., 2000)
	A. sobria	X		X	X				Wound infection	(Ashbolt et al., 1995; Jones & Wilcox, 1995; Itoh et al., 1999; Dumontet et al., 2000)
Brucella	*B. maris*	X	X			X			Neurobrucellosis, brucellosis	(Corbel, 1997; Brew et al., 1999; Foster et al., 2002; Sohn et al., 2003)
Burkholderia	*B. pseudomallei***	ND	X						Melioidosis (Nonmarine acquired)	(Hicks et al., 2000)
Campylobacter	*C. lari**	X		X					GI	(Endtz et al., 1997)
	*C. jejuni**	X		X				<500 cells (**)	GI	(Abeyta et al., 1993)
Clostridium	*C. botulinum* (type E)	X		X				0.1-1ug toxin (*) 10^5 cells (**)	Botulism, GI	(Huss, 1980; Weber et al., 1993)

(*continued*)

Table 2.1. (*Continued*)

Genus	Species[a]	Hosts of marine disease: Humans	Hosts of marine disease: Marine animals	Observed routes of human infection[b]: Seafood	Observed routes of human infection[b]: Sea water	Observed routes of human infection[b]: Zoonoses	Observed routes of human infection[b]: Aerosols	Estimated infectious dose[c]	Human syndrome	References
	C. perfringens	X	X	X				10^6–10^8 CFU/g (*)	GI	(Feldhusen, 2000; Aschfalk & Muller, 2001)
Edwardsiella	*E. tarda*	X			X				GI, wound infection, sepsis	(Kusuda & Kawai, 1998; Slaven et al., 2001)
Enterobacter	*E. cloacae*	ND							Sepsis, meningitis (Nonmarine acquired)	(Salas & Geesey, 1983; Grimes, 1991)
Erysipelothrix	*E. rhusiopathiae*	X			X	X			Skin infection, "seal finger"	(Brooke & Riley, 1999; Fidalgo et al., 2000; Lehane & Rawlin, 2000)
Escherichia	*E. coli**	X	X	X	X			10^1–10^8 cells (**)	GI	(Kueh et al., 1992; Raidal et al., 1998; Feldhusen, 2000)
Francisella	*F. philomiragia*	X			X		X		Near-drowing pneumonia	(Wenger et al., 1989; Ender & Dolan, 1997)
Halomonas	*H. venusta*	X			X	X			Wound infection	(von Graevenitz et al., 2000)
Klebsiella	*K. pneumoniae*	X			X		X		Pneumonia, wound infection	(Kueh et al., 1992; Ritter et al., 1993; Ender & Dolan, 1997)
	K. oxytoca	ND		X					Histamine production	(Lopez-Sabater et al., 1996)
Lactococcus	*L. garvieae*	ND	X						Endocarditis (nonmarine acquired)	(Fefer et al., 1998; Kusuda & Kawai, 1998) (Ravelo et al., 2003)
Legionella	*L. pneumophila*	ND					X	10^5 to 10^6/mL (Fliermans et al., 1981)	pneumonia, fever, wound infection	(Fliermans et al., 1981; Ortizroque & Hazen, 1987; Grimes, 1991)
	L. bozemanii	X			X		X		Near drowning pneumonia	(Losonsky, 1991)

Leptospira	*L. interrogans***	X	X		X	X	X	Wound, Respiratory, Leptospirosis (primarily freshwater)	(Thomas & Scott, 1997; Tryland, 2000; Levett, 2001; Arzouni et al., 2002)
	Spp.**	ND	X					Leptospirosis (Nonmarine acquired)	(Gulland et al., 1996; Levett, 2001; Colagross-Schouten et al., 2002)
Listeria	*L. monocytogenes**	X		X				Flu-like symptoms	(Colburn et al., 1990; Dillon et al., 1994)
Morganella	*M. morganii*	ND		X				Histamine production	(Lopez-Sabater et al., 1996)
Mycobacterium	*M. tuberculosis*	ND	X			X	X	10 cells (**) Tuberculosis	(Bernardelli et al., 1996; Dobos et al., 1999; Lehane & Rawlin, 2000; Montali et al., 2001)
	M. bovis	X	X			X	X	10 cells (**) Tuberculosis	(Thompson et al., 1993; Bernardelli et al., 1996)
	M. marinum	X	X		X	X		Wound, "fish tank granuloma"	(Dobos et al., 1999; De la Torre et al., 2001)
	M. haemophilum	X			X	X		Wound from coral injury	(Saubolle et al., 1996; Dobos et al., 1999; Smith et al., 2003)
Mycoplasma	*M. phocacebrale*	X	X			X		Skin, "seal finger"	(Stadtlander & Madoff, 1994; Baker et al., 1998)
Photobacterium	*P. damsela*	X	X		X	X		Wound, sepsis	(Fraser et al., 1997; Kusuda & Kawai, 1998; Rodgers & Furones, 1998; CDC, 1999; Barber & Swygert, 2000)
Plesiomonas	*P. shigelloides*	X		X				GI	(Gonzalez et al., 1999; Oxley et al., 2002; Chan et al., 2003)
Pseudomonas	*P. aeruginosa*	X			X			Skin, wound, ear infection, "diver's hand"	(Erickson et al., 1992; Ritter et al., 1993; Ahlen et al., 2000)

(*continued*)

Table 2.1 (*Continued*)

Genus	Species[a]	Hosts of marine disease		Observed routes of human infection[b]				Estimated infectious dose[c]	Human syndrome	References
		Humans	Marine animals	Seafood	Sea water	Zoonoses	Aerosols			
Rhodococcus	*R. equi*	ND	X						Wound/respiratory infection, sepsis	(Prescott, 1991; Weinstock & Brown, 2002)
Salmonella	*S. enteritidis***	X		X	X	X			GI	(Dalsgaard, 1998; Polo et al., 1999; Tryland, 2000; Aschfalk et al., 2002)
	*S. spp***	X	X	X	X	X		10^2 CFU/g varies (*)	GI	(Tryland, 2000; Aschfalk et al., 2002)
Serratia	*S. liquefaciens*	ND	X						Sepsis	(Starliper, 2001) (Grohskopf et al., 2001)
Shewanella	*S. putrefacience*	X			X				Wound infection, sepsis	(Dominguez et al., 1996; Iwata et al., 1999; Leong et al., 2000; Vogel et al., 2000; Pagani et al., 2003)
	S. alga	X			X				Sepsis, ear infection	(Nozue et al., 1992; Holt et al., 1997; Gram et al., 1999; Iwata et al., 1999)
Shigella	*S. dysenteriae**	X		X	X			10–200 cells (**)	GI	(Kueh et al., 1992; Feldhusen, 2000)
Staphylococcus	*S. aureus**	X		X	X			10^5–10^6 CFU/g (*)(oral)	GI, wound, ear, skin infections	(Charoenca & Fujioka, 1993; Thomas & Scott, 1997; Feldhusen, 2000; Tryland, 2000)
Streptococcus	*S. iniae*	X	X			X			Skin, wound infections	(Lehane & Rawlin, 2000) (Thomas & Scott, 1997) (Weinstein, 2003)

Vibrio	*V. alginolyticus*	X	X	X	X	X		Wound/ear infections, Sepsis	(Howard & Bennett, 1993; CDC, 1999, 2000)
	V. carchariae	X	X		X	X		Wound infection	(Pavia et al., 1989; Lee et al., 2002; Nicolas et al., 2002)
	V. cholerae O1	X		X	X		10^6–10^{10} cells (**)	GI, sepsis	(CDC, 1999; Lipp et al., 2002)
	V. cholerae non-O1	X		X	X	X		GI, wound/ear infection, sepsis	(Howard & Bennett, 1993; CDC, 1999, 2000)
	V. cincinnatiensis	X		X	X			Sepsis	(Brayton et al., 1986)
	V. fluvialis	X		X	X			GI	(Howard & Bennett, 1993; CDC, 1999, 2000)
	V. furnissii	X		X	X			GI	(Dalsgaard et al., 1997)
	V. hollisae	X		X	X			GI, sepsis	(Howard & Bennett, 1993; CDC, 1999, 2000)
	V. metschnikovii	X	X	X	X			GI	(Buck, 1991; CDC, 2000)
	V. mimicus	X		X	X	X		GI, wound/ear infection	.(CDC, 1999, 2000)
	V. parahaemolyticus	X	X	X	X		$\sim 10^6$ cells (**)	GI, wound/ear infection, sepsis	(CDC, 1999, 2000)
	V. vulnificus	X	X	X	X	X	10^3 to 10^5 CFU/g (Jackson, 1997)	GI, wound/ear infection, sepsis	(Howard & Bennett, 1993; Howard & Burgess, 1993; CDC, 1999, 2000; Johnson & Arnett, 2001)
Yersinia	*Y. enterocolitica**	X		X	X		10^7–10^9 CFU/g (*)	GI	(Feldhusen, 2000)

[a] Marine-indigenous species, unless otherwise indicated; *marine-contaminant from anthropogenic or natural sources, **marine source not determined.
[b] Routes of human marine-acquired disease, except for aerosol inhalation where nonmarine aerosol transmission was noted.
[c] Infectious doses are indicated as reported, either as total cells or as an environmental concentration. References indicated *Feldhusen (2000) or **PHAC (2001).

A large majority of known marine pathogens belong to the gamma-Proteobacteria. Within these, the genus *Vibrio* alone contains 11 recognized human pathogens including *V. cholera*, the etiological agent of epidemic cholera, and the hazardous seafood poisoning agents *V. vulnificus* and *Vibrio parahaemolyticus*. Many more vibrios are associated with diseases in marine animals, and only a handful of the 40 or more species currently described within the genus appear to be benign. Other notable gamma-proteobacterial pathogens are members of the *Aeromonas* and *Shewanella* genera, which are also widely distributed throughout marine environment. The proportion of marine human pathogenic species within the gamma-Proteobacteria is in contrast to terrestrial environments where groups such as the alpha-Proteobacteria and the spirochetes also contain many pathogenic members. Such discrepancy could reflect differing evolutionary trajectories of marine and terrestrial communities, or could reflect preferential culturability of gamma-proteobacterial pathogens as has generally been observed for heterotrophic gamma-Proteobacteria from the marine environment (e.g. Eilers et al., 2000b).

The deeply branching lineages of the Gram-positive bacteria also contain a high diversity of recognized marine pathogens. The *Mycobacterium* group is represented with several notable human pathogens including agents of tuberculosis, skin disease, and an expanding diversity of fish and marine mammal pathogens (Saubolle et al., 1996; Kusuda & Kawai, 1998; Dobos et al., 1999; Rhodes et al., 2001). Other Gram-positive human pathogens found in associations with marine environments include members of the *Clostridia*, *Listeria*, *Rhodococcus*, *Streptococcus*, and Mycoplasma group (Table 2.1).

2.2.2. Environmental Associations

Marine pathogens are often found in association with the surfaces of marine animals, phytoplankton, sediments and suspended detritus. The association of pathogens with marine biota has been compared to vector-borne disease in terrestrial environments as variability in environmental conditions can affect both the vector distribution and pathogen growth (Lipp et al., 2002). For example, algal and zooplankton blooms can promote proliferation of associated bacterial communities by providing microenvironments favoring growth and by exuding nutrients into the water (Lipp et al., 2002). Associations between zooplankton and pathogenic *Vibrio* and *Aeromonas* species have been observed (Kaneko and Colwell, 1978; Colwell, 1996; Dumontet et al., 2000; Heidelberg et al., 2002a) and the dynamics of attached pathogenic *Vibrio* species and *Vibrio* mediated disease (i.e. cholera) have been correlated to seasonal algal and zooplankton blooms (Kaneko and Colwell, 1978; Colwell, 1996; Heidelberg et al., 2002a).

Association with larger marine animals also influences the abundance of pathogens in the environment through activities including bioconcentration, fecal contamination, and by creating conditions favoring growth. Marine sediments with high overlying fish abundance have been found to be enriched in *Clostridium botulinum* spores suggesting deposition (Huss, 1980) while sediments underlying farmed mussels have been observed to support an enriched presence of vibrios relative to surrounding environments, possibly due to stimulated *Vibrio* growth in an organic-enriched environment (La Rosa, 2001). Filter-feeding shellfish are effective bioconcentrators of small particles and pathogenic contaminants in marine environments. Shellfish samples have been observed to harbor marine contaminants including Enteric bacteria (Burkhardt et al., 1992), *Campylobacter* (Abeyta et al., 1993; Endtz et al., 1997) and *Listeria* species (Colburn et al., 1990) in addition to potentially pathogenic indigenous flora

including *Vibrio* species (Olafsen et al., 1993; Lipp and Rose, 1997) thus, it is not surprising that shellfish have long been recognized as a potential source for marine-acquired illness.

Active growth of certain marine pathogens may occur only in association with nutrient-rich environments such as animal guts or organic-rich sediments. Such populations, when dislodged, may occur as inactive transients in seawater and act as seed populations for inoculating new habitats (Ruby and Nealson, 1978). This life-cycle has been suggested for certain fish-associated vibrios based on their ability to grow rapidly in response to nutrient addition even after prolonged incubation in seawater under starvation conditions (Jensen et al., 2003). Gastrointestinal tracts of marine animals have been shown to harbor a wide diversity of organisms closely related to bacterial pathogens (MacFarlane et al., 1986; Oxley et al., 2002). Similarly, organisms commonly associated with sediment environments include enteric pathogens (Grimes et al., 1986), and members of the genera *Vibrio* (Watkins, 1985; Hoi et al., 1998; Dumontet et al., 2000), *Aeromonas* (Dumontet et al., 2000), *Shewanella* (Myers and Nealson, 1990), *Clostridia* (Huss, 1980) and *Listeria* (Colburn et al., 1990).

Intracellular associations of bacteria with protozoan and algal hosts have been described in natural and clinical settings and may represent an additional source of pathogens in marine environments. Colonization of amoeboid hosts has been observed for several human bacterial pathogens including *Mycobacterium* (Cirillo et al., 1997; Steinert et al., 1998), *Burkholderia* (Michel and Hauroder, 1997; Marolda et al., 1999; Landers et al., 2000) and *Legionella* species (Cianciotto and Fields, 1992; Fields, 1996). *Legionella pneumophila* can replicate inside amoebas in natural waters and it is currently held that adaptation to the intracellular environment of a protozoan host predisposed *L. pneumophila,* the agent of Legionnair's disease, to infect mammalian cells (Cianciotto and Fields, 1992; Fields, 1996; DePaola et al., 2000; Harb et al., 2000; Swanson and Hammer, 2000). Relatively high concentrations of *L. pneumophila* have been found in fresh water and coastal systems (10^2 to 10^4 CFU per ml) (Fliermans et al., 1981; Ortizroque and Hazen, 1987; Fliermans, 1996). Survival of free-living *L. pneumophila* in seawater over several days has been demonstrated (Heller et al., 1998); however, extracellular growth in natural water has not been observed (Steinert et al., 1998; Swanson and Hammer, 2000). Whether associations of *Legionella* spp. or other marine pathogens with protozoan hosts promotes growth of these bacteria in marine environments remains to be determined.

Algal cells have been shown to harbor intracellular bacterial associations (Biegala et al., 2002) and it is currently debated whether agents of harmful algal blooms (HAB) maintain bacterial symbionts that participate in toxin production (Gallacher and Smith, 1999). Bacteria found in association with cultures of HAB algae have been reported to produce a level of toxin per cell volume that is equivalent to the production of toxin in the alga (Gallacher and Smith, 1999). In addition, autonomous toxin production by free-living bacteria has been observed under marine conditions (Michaud et al., 2002). The relative contribution to toxin production during HABs by free-living, surface associated, or intracellular bacteria is an area of active investigation (Carmichael, 2001; Vasquez et al., 2001; Smith et al., 2002) (see also Chapter 10 of this book). Overall, the role of protist and algal hosts for harboring marine pathogens in the environment remains an important but poorly understood factor to be considered in risk assessment.

2.2.3. Abiotic Factors

Environmental parameters such as salinity, temperature, nutrients, and solar radiation influence the survival and proliferation of pathogens directly by affecting their growth and death

rates and indirectly through ecosystem interactions. The survival of contaminant pathogens in marine environments has been shown to decrease with elevated sunlight (Rozen & Belkin, 2001; Fujioka & Yoneyama, 2002; Hughes, 2003), high salinity (Anderson et al., 1979; Sinton et al., 2002), and increased temperature (Faust et al., 1975). However, elevated nutrients and particle associations have been shown to promote the survival of marine contaminants (Gerba & McLeod, 1976). There is increasing evidence that many pathogens found as pollutants in marine environments can survive harsh environmental conditions for prolonged periods of time in a spore-like, "viable but nonculturable" (VBNC) state (e.g. Grimes et al., 1986; Rahman et al., 1996; Rigsbee et al., 1997; Steinert et al., 1997; Cappelier et al., 1999a, 1999b; Besnard et al., 2000; Asakura et al., 2002; Bates et al., 2002). The effects of environmental parameters on the survival of enteric bacteria are reviewed in detail in Chapter 10 of this book.

In contrast to microbial contaminants, marine-indigenous pathogens are adapted to prevalent environmental conditions and their proliferation may be triggered by specific factors. For example, warm water temperatures appear to have a positive effect on the abundance of human-invasive pathogens, which tend to have mesophilic growth optima. In temperate environments, the distribution of such pathogens is typically seasonal with peaks in both environmental abundance and human infection occurring during the warmer months. This has been demonstrated for human pathogenic *Aeromonas* spp. (Kaper et al., 1981; Burke et al., 1984), *Shewanella algae* (Gram et al., 1999) and vibrios (CDC, 1999, 2000; Heidelberg et al., 2002b; Thompson et al., 2004b), including *V. cholerae* (Jiang & Fu, 2001) *V. parahaemolyticus* (Kaneko & Colwell, 1978), and *V. vulnificus* (Wright et al., 1996). In addition, elevated sunlight can stimulate growth of marine indigenous heterotrophic bacteria by increasing nutrient availability by photochemical breakdown of complex polymers to release organic metabolites (Chrost & Faust, 1999; Tranvik & Bertilsson, 2001). Nutrient enrichment in seawater samples and sediments has been correlated to increases in the relative abundance of *Vibrio* populations (Eilers et al., 2000a; La Rosa et al., 2001). It remains to be established whether stimulated growth of opportunistic invasive pathogens, in response to nutrient enrichment, is a general feature of seawater environments.

2.3. ROUTES OF TRANSMISSION

Transmission of pathogens to humans through marine environments most frequently occurs by eating contaminated seafood, but can also follow other routes including seawater contact or exposure to marine aerosols and zoonoses. The potential for contracting human diseases through marine environments depends on several factors including the susceptibility of the human host, the degree of exposure to a pathogen population, and the virulence of the pathogenic agent. Individuals with medical conditions such as liver disease and diabetes, or who are immunocompromised, are most susceptible to infections (Howard & Bennett, 1993; Howard & Burgess, 1993); however, infections also occur in healthy individuals. The degree of host exposure to a marine pathogen varies with the route of transmission and has been correlated to both the environmental concentration of the pathogen and the duration of exposure. For the purposes of risk assessment for seafood consumption, an average amount of ingested seafood is assumed (e.g., 110 g oyster meat (Miliotis et al., 2000)) and swimming related illnesses have been correlated to time spent in the water (Corbett et al., 1993). However, no explicit models appear to have been formulated for prediction of other routes of exposure (e.g., animal

contact, or aerosol inhalation). Finally, the virulence of the pathogenic population determines the dose needed to establish human disease. In several cases, it has been observed that strains most closely resembling clinical isolates represent only a small subset of related co-occurring organisms, suggesting that infections from marine environments may frequently be initiated by small numbers of highly virulent variants (Jackson et al., 1997).

2.3.1. Seafood Consumption

The most important route of infection by marine pathogens is by consumption of contaminated seafood resulting in symptoms from self-limiting gastroenteritis (typical seafood poisoning) to invasive infections that are potentially fatal. *Vibrio* species are the most significant risk in seafood consumption and an estimated 10,000 cases of food-borne infection occurs in the United States each year (FDA, 1994; Altekruse et al., 1997). But other bacterial genera naturally found in association with fish and shellfish have also been implicated in seafood-borne diseases (e.g., *Aeromonas*, *Clostridium*, *Plesiomonas*). Fecal contamination from human sewage or animal sources is recognized as an additional important source of seafood-borne pathogens (e.g., *Campylobacter*, *Escherichia*, *Listeria*, *Salmonella*, *Shigella*, *and Yersinia*) (Feldhusen, 2000). However, in several cases a clear distinction cannot be made whether a pathogen is a fecal contaminant or a natural part of the marine community. For example, *Salmonella*, generally considered a marine contaminant, may be a natural part of marine ecosystems (Tryland, 2000; Aschfalk et al., 2002). Other genera, such as *Campylobacter*, are detected in the feces of marine birds (Endtz et al., 1997) and could be described as "endemic contaminants" since their presence can be detected in environments not polluted by humans.

Infection by ingestion generally requires relatively large doses of pathogens (e.g., 10^5–10^{10} cells for most gamma proteobacterial pathogens), although some highly virulent pathogens such as *Shigella* or enterohemoragic *Escherichia coli* can establish infections with doses as small as 10–100 cells (PHAC, 2001) (Table 2.1). Levels of marine-indigenous pathogens in fresh seafood are usually low enough to be considered safe so that only the growth of these organisms is regarded as a hazard (e.g., during periods of improper handling) (Feldhusen, 2000). For example, nonrefrigeration of oysters after harvesting can amplify the endemic *Vibrio* population 10,000-fold (Miliotis et al., 2000) resulting in levels that are deemed unsafe for human consumption (i.e., $\geq 10^4$ cells/g oyster (FDA, 1997)).

While cooking minimizes the risk of seafood-borne infection, poisoning can occur from heat-stable bacterial toxins or compounds. Scombroid (or histamine) fish poisoning is caused when bacteria containing the enzyme histadine-decarboxylase proliferate in improperly stored fish rich in the amino acid histadine (e.g., tuna, sardines, and salmon) (Burke & Tester, 2002). Bacterial transformation of histadine can produce dangerous levels of histamine, consumption of which can lead to severe allergic reactions. Several types of bacteria including *Morganella morganii* and *Klebsiella oxytoca* have been implicated in histamine production in fish (Lopez-Sabater et al., 1996). In addition, toxins produced by marine bacterial species may be concentrated by the activities of filter feeding shellfish. Although this has not been confirmed as a route of human pathogenicity in marine environments, toxin production has been observed by bacterial strains associated with HAB algae including members of the *Roseobacter* and *Alteromonas* genera, and cyanobacterial species (Gallacher & Smith, 1999; Carmichael, 2001).

2.3.2. Seawater Exposure

Pathogens can be transmitted to humans through seawater during accidental ingestion, inhalation, or by direct exposure of ears, eyes, nose, and wounded soft tissue. Although sewage contamination has long been recognized as a significant risk factor in acquiring illnesses after seawater exposure, sewage-borne pathogens are primarily viral rather than bacterial (Cabelli et al., 1982; Griffin et al., 2001). Invasive bacterial infections acquired in marine environments have primarily been attributed to marine endemic species including gamma-proteobacterial strains related to *Aeromonas*, *Halomonas*, *Pseudomonas*, *Shewanella*, and *Vibrio* (Table 2.1). In beaches with high swimmer density, human-shed *Staphylococcus* or *Streptococcus* can cause minor wound and ear infections (Charoenca & Fujioka, 1993; Thomas & Scott, 1997). Other bacterial infections that have been reported after exposure to marine or estuarine waters include leptospirosis (Thomas & Scott, 1997) and skin granulomas caused by water-borne *Mycobacterium marinum* (Dobos et al., 1999). Near-drowning experiences in marine environments bring seawater into the lungs and can result in pneumonia (Ender & Dolan, 1997; Thomas and Scott, 1997). Such infections have been reported for marine indigenous pathogens including *Legionella bozemanii*, *Francisella philomiragia*, *Klebsialla pneumonia* and several *Vibrio* and *Aeromonas* species (Ender & Dolan, 1997).

Although the range of infectious doses for wound and skin infections is not known and the degree of exposure is difficult to estimate, the danger may potentially be high. Fifty percent mortality was observed for artificially wounded rats exposed to $\sim 10^7$ CFUs of marine and clinical isolates of *Aeromonas hydrophila*, *V. parahaemolyticus*, and *V. vulnificus* (Kueh et al., 1992). In the same study, similar mortalities were observed in rats exposed to 1 ml aliquots of seawater from multiple sites, suggesting a high degree of indigenous seawater-associated virulence (Kueh et al., 1992).

2.3.3. Aerosol Exposure

The first case of Legionnaires Disease in 1976 demonstrated the importance of airborne transmission of the water-borne bacterial pathogen *Legionella pneumophila* (McDade et al., 1977). Transmission of bacterial disease by marine aerosols has not been documented but should be considered as a potential route of infection. Studies have shown that *Mycobacterium* species are enriched in aerosols from natural waters (Wendt et al., 1980; Parker et al., 1983) and additional respiratory disease agents, which have been detected in seawater, include *F. philomiragia*, *Legionella* spp., *Acinetobacter calcoaceticus*, and *K. pneumoniae* (Grimes, 1991; Ender & Dolan, 1997). In general, infectious doses for respiratory agents are small, e.g. 5–10 organisms for *Mycobacterium tuberculosis* infection. In addition, aerosols, generated in coastal environments by wave activity, can transmit algal toxins to humans (Van Dolah, 2000) and cause viruses to become airborne (Baylor et al., 1977). Thus, marine aerosols may be an unrecognized factor in the transmission of diseases from marine environments.

2.3.4. Marine Zoonoses

Zoonoses are naturally transmissible diseases from animals to humans. Warm-blooded marine mammals harbor and are afflicted by a wide variety of pathogens posing zoonotic risk to humans including *Brucella, Burkholderia, Clostridium, Helicobacter, Mycobacterium,*

Rhodococcus, and *Salmonella* species (Bernardelli et al., 1996; Harper et al., 2000; Tryland, 2000; Aschfalk & Muller, 2001; Aschfalk et al., 2002) (Table 2.1). Tuberculosis, a chronic respiratory disease caused by *Mycobacterium* species including *M. tuberculosis* and *M. bovis*, has afflicted natural and captive populations of marine mammals (Bernardelli et al., 1996; Montali et al., 2001) and transmission from seal to man has been documented (Thompson et al., 1993). Brucellosis, a systemic infection, is transmitted to humans from infected animals, meat, or dairy products in many parts of the world. Brucellosis has also been observed in a wide range of marine animals including dolphins, porpoises, whales, seals, and otters (Tryland, 2000; Foster et al., 2002). The zoonotic potential of these marine *Brucella* species has been recognized after three incidents of infection, first of a researcher handling a marine isolate (Brew et al., 1999) and then in two cases of neurobrucellosis attributed to a marine *Brucella* strain in Peru (Sohn et al., 2003).

Injuries inflicted by marine animals or sustained during their handling are especially susceptible to infection by associated microorganisms and therefore emergency treatment of bites (e.g., from sharks, moray eels) includes broad-spectrum antibiotics (Erickson et al., 1992; Howard & Burgess, 1993). Handling of fish or crabs has been associated with infection by *Erysipelothrix rhusopathiae*, a mycoplasma-like organism common on the skin of fish, which manifests as a localized swollen purple area around a wound (fish handler's disease) (Thomas & Scott, 1997). Other mycoplasma-like organisms including *Mycoplasma phocacerebrale* have been isolated from seals during pneumonia epizootics and have been implicated in development of "seal finger," a local infection of the hands in humans (Kirchhoff et al., 1989; Stadtlander & Madoff, 1994; Baker et al., 1998).

The transmission of disease between farmed and wild fish populations is one of many concerns regarding the sustainability of aquaculture practices (Garrett et al., 1997; Naylor et al., 2000). The zoonotic potential of farmed fish environments has also been recognized on several occasions. The fish pathogen, *Streptococcus inae* (Zlotkin et al., 1998; Colorni et al., 2002), caused an outbreak of infection in fish farmers in British Columbia (Weinstein et al., 1996, 1997). Additional health hazards of fish handlers include infections with *A. hydrophila*, *Edwardsiella tarda*, *E. rhusopathiae*, *M. marinum*, and *Vibrio* species (Lehane & Rawlin, 2000). In addition, several currently emerging pathogens of fish populations are closely related to human pathogens (Fryer & Mauel, 1997; Rhodes et al., 2001; Starliper, 2001). Recently, *Serratia liquefaciens* was identified as an agent of deadly systemic hospital infections in humans (Grohskopf et al., 2001) and in the same year was identified as a pathogen of farmed Atlantic salmon (Starliper, 2001).

2.4. INDICATORS FOR MARINE RISK ASSESSMENT

The quality of marine waters has been routinely monitored using detection of indicator organisms found in association with human pollution. Indicators are elements that can be efficiently monitored to approximate the risk of human exposure to a given environment. While the indicators themselves do not necessarily cause disease, their presence in an environment suggests a high probability of co-occurring pathogens. Although traditionally indicator organisms have been relied upon for water quality assessment, the use of physical and chemical proxies and direct detection of pathogen populations are showing promise as tools for future water quality management.

2.4.1. Indicators for Sewage Pollution

Sewage-associated public health risks continue to plague coastal environments worldwide. The NRDC[1] reports that 12,184 U.S. beach closings or advisories were issued in 2002 (of 2922 reporting beaches) of which 87% were attributed to poor bacterial water quality (as monitored by indicators for fecal pollution) (Dorfman, 2003). In a landmark epidemiological study, Cabelli et al. (1982) found that illness (primarily gastroenteritis and respiratory infections) associated with swimming in several marine environments increased linearly with the degree of site pollution. They further showed that levels of Gram-positive fecal enterococci and fecal coliforms were good proxies for sewage contamination. Based upon this and similar studies the current USEPA[2] standard for acceptably safe beaches is a monthly geometric mean of 35 enterococci per 100 ml (Dufour et al., 1986) and a median of 14 fecal coliforms per 100 ml in shellfish harvesting waters (USEPA, 1988).

The use of enterococci and fecal coliform levels as indicator organisms for marine water quality assessment has been repeatedly called into question. These indicator species have shown varying degrees of specificity for detecting sewage contamination against background environmental fluctuations from animal and environmental sources (Grant et al., 2001; Boehm et al., 2002). Boehm et al. (2002) showed that coastal enterococci levels are enriched by bird activity in adjacent estuaries. Alternative sewage-borne indicators, such as *Clostridium perfringens*, have been considered due to their stability in the marine environment (Fujioka, 1997); however, they too are found in association with marine animals (e.g., Aschfalk & Muller, 2001) and may be subject to environmental variability. In addition, their correlation to human illness has not been convincing (Dufour et al., 1986). Furthermore, exclusive reliance on fecal indicator bacteria for marine water quality assessment has been challenged due to their limited ability to predict viral contamination and the presence of marine-indigenous pathogens (Dumontet et al., 2000; Tamplin, 2001). While sewage indicators remain a useful tool for monitoring water pollution, continued efforts to establish alternative indicators for nonsewage related risks hold promise for future risk assessment.

2.4.2. Indicators for Nonsewage Related Risk

Additional factors that have been related to human risks from seawater exposure include swimmer density, eutrophication, and thermal pollution. High swimmer density at bathing beaches has been correlated to the acquisition of ear and minor skin infections from human shed bacteria. Levels of the pathogen, *Staphylococcus aureus*, have been proposed as an indicator for exposure to human-shed bacteria with levels above 100 CFU per 100 ml of seawater considered unsafe (Charoenca & Fujioka, 1993; Fujioka, 1997).

Eutrophication of coastal environments may be linked to infections by marine indigenous pathogens (e.g., Kueh et al., 1992). The relative abundance of *Vibrio* populations in seawater samples increases in response to organic nutrient enrichment, and pollution from aquaculture environments has been correlated to increased proportions of vibrios in underlying sediments (Eilers et al., 2000a; La Rosa et al., 2001). Accordingly, the prevalence of vibrios or other aerobic heterotrophs has been suggested as an indicator for nutrient enrichment in marine environments (La Rosa et al., 2001).

[1] National Resources Defense Council.
[2] United States Environmental Protection Agency.

That high seawater temperature bears higher risk of exposure to marine pathogens has been established in studies of shellfish (Wright et al., 1996; Motes et al., 1998; Miliotis et al., 2000), natural waters (Wright et al., 1996; Jiang & Fu, 2001; Heidelberg et al., 2002b; Louis et al., 2003; Thompson et al., 2004b), and the incidence of epidemic cholera (Colwell, 1996; Pascual et al., 2000). Remote sensing of sea surface temperature is currently being explored as a means to predict the onset of cholera outbreaks along the Indian and Bangladesh coasts (Lobitz et al., 2000).

2.5. DETECTION AND QUANTIFICATION

In this section an overview of the methods currently available to detect, identify, and enumerate marine pathogen (or indicator) populations is presented. At the center of the discussion will be methods with proven utility for targeting specific populations within environmental microbial communities. However, several techniques used to isolate and identify marine pathogens in clinical specimens will also be briefly evaluated. Methods used to identify and quantify microbial populations can be divided into three main groups: culture, immunology, and nucleic acid based. However, protocols frequently do not fall exclusively into one category but represent combinations. Because of the considerable number of published protocol and commercial kits, this overview presents the general principles that define these three main groups of methods. Where specific examples are given these have been selected because they have been (i) employed by several laboratories and/or (ii) characterized with respect to their limits of sensitivity and specificity. A summary of representative nucleic acid- and immunology-based methods for detection or quantification of marine-relevant pathogen populations is presented in Table 2.2. In a few cases, methods are described that have not yet been applied to pathogen detection but hold potential.

Methods for monitoring pathogen populations should be selected by evaluating the factors that mediate exposure of humans to the pathogen (e.g., abundance, virulence/infectious dose, route of exposure) and the constraints of the method (e.g., sensitivity, specificity, dynamic range, cost). Methods targeting pathogen populations must be sensitive enough to monitor populations at levels below the infectious dose, and specific enough to recognize the target group without generating false positives by cross-reacting with nontarget organisms. Detection requires positive identification at or above specified threshold concentrations while enumeration requires flexibility to identify a range of population levels. For clinical purposes, detection is often sufficient, while quantification of hazardous populations is preferable for analysis of environmental samples. The methods also differ greatly in speed and cost of implementation and therefore the most accurate method may not always be the most preferable when rapid decision making is required. The following sections present our attempt to take these considerations into account while evaluating the strengths and weaknesses of various methods.

2.5.1. Culture-Based Methods

Detection of pathogens via culturing requires enrichment of a target population over other environmental bacteria. This employs selective and/or differential media, which provide a ‘presumptive identification’ and can be followed by any number of tests (e.g. biochemical, immunological or molecular) to confirm the identity of isolates. A medium is selective if it favors the growth of a specific population of organisms and is differential if it allows distinction

Table 2.2 Representative immunological and molecular methods for detection of human pathogens in environmental samples.

Bacteria	Sample	Target	Method	Sensitivity	Reference
Escherichia coli	Food	Shiga-toxin gene (*stx*)	PCR following enrichment Colony hybridization following enrichment	ND	(Dutta et al., 2000)
	Waste and drinking water	16S rRNA gene	Fish	10^7–10^8 cells/ 100 mL	(Baudart et al., 2002)
	Artificially contaminated food	NA	Real-Time PCR (BAX®kit, Dupont)	10^2–10^3 cells/100 mL	(Bhagwat, 2003)
	Water	β-D-galactosidase gene	Real-Time PCR following enrichment with magnetic beads	10^3 cells/100 mL	(Foulds et al., 2002)
	Waste water	*gcvp* gene	Multiplex PCR	ND	(Turner et al., 1997)
	Food outbreaks—clinical isolated strains	Genome	PFGE (PulseNet)	ND	(Breuer et al., 2001)
	Wastewater wetlands	*stx*1, 2 genes and intimin (*eae*) gene	Real-time PCR	W/o enrichment: 3×10^4 CFU/g With enrichment: 10 CFU/g	(Ibekwe et al., 2002)
Campylobacter spp.	Poultry	16S rRNA gene	Modified RT-PCR followed by hybridization	<1 CFU/g	(Uyttendaele et al., 1995, 1996)
	Mussels and oysters—isolated strains	GTPase gene	PCR and line-blot hybridization	ND	(Van Doorn et al., 1998)
	Water, food	VS1 sequence	Real-time PCR	6–15 CFU/reaction	(Yang et al., 2003)
	Food outbreaks—clinical isolated strains	Genome	PFGE (PulseNet)	ND	(Ribot et al., 2001)
	Water, and waste water	*fla*A, B genes	Modified PCR	3–15 CFU/ 100 mL	(Waage et al., 1999c)
Listeria monocytogenes	Food outbreaks—clinical isolated strains	Genome	PFGE (PulseNet)	ND	(Graves & Swaminathan, 2001)
	Food outbreaks—clinical isolated strains	Listeriolysin O (*hly*) gene	PCR	ND	(Jackson et al., 2000)
	Food outbreaks—clinical isolated strains	*hly* gene	Dot-blot hybridization (US-FDA established protocols)	ND	(Jackson et al., 2000)

	Food	16S rRNA gene	PCR following culture enrichment	0.04–0.2 CFU/g	(Somer & Kashi, 2003)
	Household waste samples	*iap* gene	PCR following culture enrichment or directly from the sample	W/o enrichment: 10^7 CFU/g waste sample With enrichment: <10 CFU/g waste sample	(Burtscher & Wuertz, 2003)
Staphylococcus aureus	Water/food outbreaks—clinical isolates	Enterotoxin B gene	Colony hybridization (US-FDA established protocols)	ND	(Jackson et al., 2000)
	Household waste samples	*nuc* gene	PCR following culture enrichment or directly from the sample	W/o enrichment: 10^6 CFU/g waste sample With enrichment: <10 CFU/g waste sample	(Burtscher & Wuertz, 2003)
Yersinia spp.	Water/food outbreaks—clinical isolates	*ail*, *inv* genes	Colony hybridization (US-FDA established protocols)	ND	(Jackson et al., 2000)
	Raw meat	Enterotoxin *yst* gene	Real-time PCR	10^6 CFU/g	(Vishnubhatla et al., 2001)
	Water/food outbreaks—clinical isolates	Virulence plasmid *vir*F gene	Dot-blot hybridization—(US-FDA established protocols)	ND	(Jackson et al., 2000)
	Household waste samples	16s rDNA gene	PCR following enrichment or directly from the sample	w/o enrichment: 10^7 CFU/g waste sample/ with enrichment: <10 FU/g waste sample	(Burtscher & Wuertz, 2003)
	Water and waste water	*yad* gene	PCR following culture enrichment	8–17 CFU/ 100 mL	(Waage et al., 1999a)

(*continued*)

Table 2.2 (*Continued*)

Bacteria	Sample	Target	Method	Sensitivity	Reference
Shigella spp.	Sewage polluted seawater	Invasion plasmid antigen H (*ipa*H)	PCR	10 to 100 CFU/reaction	(Kong et al., 2002)
	Water	*ipa*BDC, *ipa*H genes and *stx*1 gene	PCR	5×10^2 CFU/100 mL	(Faruque et al., 2002)
Salmonella spp.	Food (outbreak)	*inv*A gene	Real-time PCR	ND	(Daum et al., 2002)
	Waste samples	*omp*C gene	PCR following enrichment or directly from the sample	w/o enrichment: 10^7 CFU/g waste sample/with enrichment: <10 CFU/g waste sample	(Burtscher & Wuertz, 2003)
	Water and shellfish	*inv*A gene	PCR	3×10^5 cells/100 ml	(Dupray et al., 1997)
	Sewage polluted seawater	*ipa*B gene	PCR	10 to 100 CFU/reaction	(Kong et al., 2002)
	Water, and waste water	ST11, 15 sequences	PCR	10 CFU/100 ml	(Waage et al., 1999b)
	Harp seals	LPS	ELISA	ND	(Aschfalk et al., 2002)
Vibrio cholerae	Raw oysters	Hemolysin (*hly*A)	Real-Time PCR	10^2 CFU/100 mL	(Lyon, 2001)
	Waste waters estuarine waters—environmental isolates	*ctx*A, *hly*A, *omp*U, *stn/sto*, *tcp*A, *tcp*I, *tox*R, and *zot* genes	Multiplex PCR	ND	(Rivera et al., 2001)
	Water/food outbreaks—clinical isolates	Cholera enterotoxin gene (*ctx*)	ELISA (US-FDA established protocols)	ND	(Jackson et al., 2000)
	Sewage polluted seawater	*ctx* gene	PCR	10–100 CFU/reaction	(Kong et al., 2002)
	Seawater	ctxA, 16s–23s rRNA	PCR	ND	(Lipp et al., 2003)
	Estuarine water	O1 or O139 surface antigens	Agglutination following culture enrichment	2×10^3 CFU/mL ND	(Hasan et al., 1995; Louis et al., 2003)
	Seawater	16s–23s rRNA	Colony hybridization following enrichment	ND, but <100 CFU 100 mL was detected	(Jiang & Fu, 2001)
	Seawater where *V. cholera* is endemic (Bangladesh)	O1 or O139 LPS antigens	Immunofluorescence microscopy	$1.5–4 \times 10^4$ cells/100 mL	(Brayton et al., 1987; Hasan et al., 1995)

V. parahaemolyticus	Raw oysters	Thermostable direct hemolysin (*tdh*) gene	Colony hybridization following culture enrichment	10 CFU/g D	(DePaola et al., 2000; Nordstrom & DePaola, 2003)
	Raw oysters	*tdh* gene	Real-time PCR following culture enrichment	1 CFU/reaction	(Blackstone et al., 2003)
	Sewage polluted seawater	16s–23s rRNA	PCR	10–100 CFU/reaction	(Kong et al., 2002)
	Seawater	*tox*R, *tdh*, *trh* genes	Most-probable-number PCR	3 cells/100 mL	(Alam et al., 2003)
	Seafood	*tdh* gene	PCR with enrichment	0.3 cells/g	(Hara-Kudo et al., 2003)
V. vulnificus	Seawater, sediment, fish	Cytolysin gene	Hybridization following culture enrichment	2 CFU/100 mL	(Hoi et al., 1998)
	Oysters—isolated strains	Genome	PFGE	ND	(Jackson et al., 1997; Tamplin et al., 1996)
	Eels	LPS	ELISA	10^4–10^5 cells/reaction	(Biosca et al., 1997)
	Water	Hemolysin (*vvhA*) gene	Colony hybridization	2×10^3/100 mL	(Lee et al., 2001)

NA: not applicable.

of specific properties of the target population. Formulations of media designed to isolate specific organisms have been widely published in the literature and are available through various microbiological handbooks [e.g. (Atlas, 1995)]. For example, bacteria in the genus *Vibrio* (a marine-endemic genus containing a high diversity of human and animal pathogens) can be readily isolated using thiosulfate-citrate bile salts sucrose (TCBS) media where selectivity for organisms tolerant of both intestinal and marine environments (characteristic of vibrios) is provided by the combination of bile salts and alkaline pH, respectively. The TCBS medium is also differential for the trait of sucrose fermentation because it contains an indicator dye, which responds to acid produced by sucrose fermentation during growth. TCBS media has been routinely employed in clinical settings to diagnose gastrointestinal diseases, seafood poisoning, or wound infections mediated by *Vibrio* species. For diagnosis of the diarrheal disease cholera, presumptive identification of the etiological agent *V. cholerae* as small yellow colonies on TCBS media must be confirmed with subsequent tests, as certain marine vibrios and alkaline-tolerant enteric bacteria can manifest similar morphologies on the media (Lotz, 1983). However, the specificity for the target group (e.g. vibrios) of such media can be surprisingly good. For example, >95% of seawater isolates grown on an improved formulation of TCBS (2–3% salt) (Toro et al., 1995) were *Vibrio* sp. as determined by 16S rRNA sequencing and the remainder were closely related genera (Thompson et al., 2005). Similarly, a selective media designed for presumptive identification of *V. vulnificus* by combination of antibiotic resistance, metabolism of cellobiose, and colony morphology, yielded 79% specificity for target organisms as confirmed by hybridization with DNA probes (Hoi and Dalsgaard, 2000).

Growth-based quantification of pathogen abundance has long tradition and is often referred to as direct viable counts (DVC). Abundance is either inferred from the number of colony forming units (CFUs) on culture plates or by Most Probable Number (MPN) dilutions of environmental samples. However, to ensure the accuracy of detection, representative presumptive positive strains must be corroborated by more extensive characterization with biochemical tests or molecular assays (described in the next sections). The dilution or concentration (e.g., by filtration) of samples prior to culture-based enumeration can accommodate a wide dynamic range of environmental microbial population sizes. Protocols for culture-based enumeration of marine pathogens include those for *Aeromonas* (Villari et al., 1999), *Clostridium* (Glasby and Hatheway, 1985),*Legionella* (Boulanger and Edelstein, 1995; Bartie et al., 2003), *Vibrio* sp. (Hernandezlopez et al., 1995) and *V. vulnificus* (Hoi and Dalsgaard, 2000; Cerda-Cuellar et al., 2001).

A disadvantage of culture-based detection and enumeration methods is the dependence on reproducible and quantitative growth of target pathogen populations on culture media. Indeed, the majority of natural bacteria have been shown to be inherently difficult to culture and even those that are typically easy to culture can enter stages where their culture efficiency drops dramatically. For example, certain pathogens have been shown to enter a viable but non-culturable state (VBNC) in response to shifts in environmental conditions, complicating the interpretation of population dynamics observed in culture-based studies (Grimes et al., 1986; Rahman et al., 1996; Rigsbee et al., 1997; Steinert et al., 1997; Cappelier et al., 1999a; Cappelier et al., 1999b; Besnard et al., 2000; Asakura et al., 2002; Bates et al., 2002). Thus, it is important to evaluate whether non-culturable states have been described for the target pathogens and to take these into account in the evaluation of protocols.

An additional limitation of culture-based techniques is the rate at which the target population grows to detectable levels. Several assays designed for routine monitoring of marine water

quality have been optimized for speed. For example, detection and enumeration of Fecal Enterococci using USEPA Method 1600 requires a 24 hr incubation for presumptive results, which are then verified by biochemical testing over an additional 48 hours. However, with notable exceptions, most culture-based identification schemes for specific populations are time and labor-intensive, and may require preliminary enrichment or decontamination steps that confound enumeration. For example, pathogenic *Mycobacteria* species grow relatively slowly in culture (1 to >20 weeks) and thus can easily be overgrown by faster-growing organisms. Since *Mycobacteria* are resistant to harsh conditions (i.e. alkaline and acidic treatments), washing environmental samples at high or low pH can be coupled with selective media to eliminate faster growing competitors and increase the efficiency of their isolation (Songer, 1981) (Hartmans and DeBont, 1999).

Despite some disadvantages of culture-based methods, including the variability in culturing efficiency of target populations and the labor intensive nature of microbial cultivation, significant benefits remain. Most notably, the cost of materials needed for culture-based assays is often less than for molecular methods, which can require extensive training, and highly specialized materials and equipment. In addition, cultured isolates allow subsequent investigations into the virulence and/or clinical significance of environmental pathogen populations.

2.5.2. Immunological Methods

Immunological detection has been used to identify and in some cases enumerate pathogen populations in clinical and environmental samples. These methods rely on the inherently high specificity of immune reactions and typically target pathogen-specific antigens such as cell-wall lipopolysaccharides (LPSs), membrane and flagellar proteins or toxins. Immunoassays can be categorized into three main groups: enzyme-linked immunosorbent assay (ELISA), immunofluorescent microscopy, and agglutination assays. These have been essential diagnostic tools in medicine and food quality monitoring because they are fast and accurate (for a detailed description see Schloter et al., 1995; Rose et al., 2002).

There are several notable challenges for the implementation of immunological methods to detection of pathogens in environmental samples, which contain a large diversity of unknown bacteria. First, the sensitivity of many current methods is not high enough for detection of pathogens at low, environmentally relevant, concentrations. Second, false positive results can be generated by cross-reaction of antibodies with antigens of similar but nontargeted organisms. This is particularly problematic when polyclonal antibodies are used since these are complex mixtures of antibodies against multiple, mostly uncharacterized cell structures. However, the increased facility with which antibodies specific for single antigenic determinants (monoclonal antibodies) can be produced is improving the specificity of assays (Schloter et al., 1995; Mitov et al., 2003). Finally, design and production of specific antibodies generally requires growth of target microorganisms, constraining the applicability of the methods to culturable populations. Despite these limitations, immunological methods have many potential applications for detection of pathogens in clinical and environmental settings.

2.5.2.1. Enzyme-Linked Immunosorbent Assay

Several ELISA assays have been developed for identification of marine-pathogen populations in human or animal clinical samples. For the indirect ELISA assay, bacteria (or

bacterial antigens) are immobilized in microtiter wells and are challenged with pathogen-specific antibodies. These antibodies can be contained in anti-sera collected from infected individuals or laboratory animals (polyclonal) or can be derived from clonal cell lines (monoclonal). In the direct ELISA assay, antibodies linked to microtiter plates are challenged with antigens (e.g., bacterial cells). In both assays, detection of positive antigen–antibody complexes is accomplished by activation of an enzyme reporter system (e.g., alkaline phosphatase, peroxidase or β-galactosidase) upon binding. This typically results in formation of colored product, which can be measured. A considerable number of ELISA assays are available for pathogen-specific antigens including the LPS of *Salmonella* (House et al., 2001), the cholera toxin antigen of *V. cholerae* (Jackson et al., 2000), the heat-labile enterotoxin of enterotoxigenic *E. coli* (Germani et al., 1994b; Koike et al., 1997), the Shiga-like toxin I of diarrhoeogenic *E. coli* (Germani et al., 1994a), and the listeriolysin O and internalin A of *Listeria monocytogenes* (Jackson et al., 2000; Boerlin et al., 2003; Palumbo et al., 2003). Many of these assays are commercially available, are routinely applied to clinical specimens or contaminated food samples, and possess high potential for automation.

The sensitivity achieved by most ELISA assays makes them useful for clinical detection of pathogens, and in some cases quantification. However, the application of the ELISA assay to environmental samples frequently requires careful evaluation and optimization due to the generally low concentration of pathogens. For example, a direct ELISA assay for *V. vulnificus* was evaluated in artificially infected eel and water samples (Biosca et al., 1997). Antibodies targeted against biotype 2 LPS yielded a detection limit of 10^4–10^5 cells per well, corresponding to water-borne *V. vulnificus* populations near 10^6 CFU per ml (Biosca et al., 1997). Because typical environmental concentrations do not exceed 10^3 cells per ml the assay was not adequate for *V. vulnificus* detection in natural seawater. ELISA assays have been successfully used in several studies to characterize the pathogen populations present in marine mammals. This included detection of *Salmonella* spp. LPS (Aschfalk et al., 2002) and *C. perfringens* toxin (Aschfalk & Muller, 2001) in seal populations of the Greenland Sea, and *Brucella* populations in marine mammals in North Atlantic coastal waters (Tryland et al., 1999; Foster et al., 2002).

2.5.2.2. Immunofluorescence Microscopy

Immunofluorescence has been used to identify and quantify marine pathogens in environmental, food, and clinical samples. In these assays, fluorescence-conjugated antibodies are incubated with fixed samples (e.g., cell suspensions, filter concentrated cells, or tissue sections) and positive reactions are detected by epifluorescent microscopy. Pathogen-specific antibodies can be conjugated directly to a fluorescent marker (e.g., fluorescein isothiocyanate (FITC) or Texas Red), or can be targeted by a second, fluorescently labeled antibody. When samples are prepared quantitatively, enumeration of positive reactions provides a measure of population size. Indeed, several examples highlight the sensitive detection of water- and food-borne pathogen populations. *E. coli* abundances in seawater were detected above 1 cell per ml by applying a primary polyclonal mixture followed by a secondary, FITC-conjugated antibody to filter concentrated samples (Caruso et al., 2000, 2002). Similarly, FITC-conjugated monoclonal antibodies targeting *V. cholerae* O1 or O139 detected between 10^2 and 10^4 cells per ml in filter-concentrated river and estuarine waters in Bangladesh (Brayton et al., 1987; Hasan et al., 1995). *L. pneumophila* abundance in lake water was determined over a range of 9–3000 cells per ml by a direct immunofluorescence assay with monoclonal antibodies against serogroups 1–4 following 500-fold concentration of samples by centrifugation (Fliermans et al., 1981). For

routine detection of pathogens in food and clinical samples a number of fluorescently conjugated antibodies are commercially available. For example, polyclonal *Salmonella* spp. antibody mixture, directly conjugated with Texas-Red, allowed the detection of *Salmonella* spp. in fresh and processed meats (Duffy et al., 2000). These examples illustrate that immunofluorescence holds promise for sensitive and accurate detection of pathogens in environmental samples.

2.5.2.3. Agglutination Assays

Agglutination assays are routinely used for identification of clinical isolates and have in some cases been applied to detection of environmental pathogens. The assay is based on antigen binding to antibodies that are linked to particles (e.g., latex beads). Antibody–antigen aggregates result in the formation of visible clumps that are easily observed on a microscope slide or in a liquid test tube format. Several agglutination kits are commercially available including diagnostic tests for *S. aureus* (targeting protein A and clumping factor) (Wilkerson et al., 1997) and the BengalScreen agglutination test, which has been shown to identify *V. cholerae* O139 above 2×10^3 CFU per ml in clinical and environmental samples (Hasan et al., 1995). Additional agglutination assays have been developed targeting the LPS and outer membrane proteins of *Pseudomonas anguilliseptica* (Lopez-Romalde et al., 2003), *Brucella bacteremia* (Almuneef & Memish, 2003), and *Salmonella* spp. (Jackson et al., 2000). The greatest advantage of agglutination assays is that they are relatively simple, rapid, and inexpensive yet retain the potentially high specificity of immunological methods. However, as with most immunological methods, the sensitivity needs to be carefully evaluated for environmental applications.

2.5.3. Nucleic-Acid-Based Methods

Advances in molecular biology have revolutionized clinical and environmental microbiology by facilitating the identification of emerging pathogens, the detection of environmental populations, and the discrimination between closely related pathogenic and nonpathogenic bacteria. Molecular methods allow the characterization of bacteria by genotype rather than by phenotype and thus require identification of a unique genetic signature for individual or groups of pathogenic strains. Determination of genetic signatures remains the biggest challenge and typically requires extensive sequence characterization of the pathogen and related bacteria. However, if specific signatures can be identified, molecular methods provide a powerful diagnostic tool because nucleic acids can be rapidly and sensitively measured.

Discrimination of nucleotide variation among genes, whose occurrence is specific to an organism or whose sequence differentiates organisms, is often achieved by nucleic acid hybridization; other methods rely on restriction cutting of the chromosome. Hybridization-based methods include fluorescence in situ hybridization (FISH) and filter hybridization (e.g., colony and dot-blot hybridization), and the polymerase chain reaction (PCR). The PCR couples hybridization of short DNA molecules (primers) to template molecules followed by amplification with a polymerase (see below). Molecular typing methods have used PCR (e.g., multilocus sequence typing (MLST)) or restriction cutting (e.g., pulsed field gel electrophoresis (PFGE)) for analyzing genomic signatures. The general principles of hybridization-based, PCR-based, and molecular typing methods have been reviewed in widely available protocol books (Sambrook & Russel, 2001; Persing, 2003).

Important considerations for development of hybridization-based or PCR-based pathogen detection assays are those of probe specificity and sensitivity in the choice of target genes.

Short probes (oligonucleotides) can be hybridized with the highest specificity since they can differentiate as little as a single nucleotide change between targets; however, they can only carry a limited amount of label so that their detection limit is relatively high. On the other hand, longer probes (polynucleotides) can carry multiple labels but cannot distinguish closely related sequences because mismatches up to a certain level cannot be differentiated. Thus, knowledge of sequence variation among genes in related pathogenic and nonpathogenic strains is important for judgment of specificity. For environmental pathogens this remains a challenge since it has been shown that very similar pathogenic and nonpathogenic strains can coexist (Zo et al., 2002). Furthermore, genome sequencing has demonstrated that pathogenicity has frequently arisen via transfer of genes from other bacterial groups, and even genes, which are unique to a pathogen among closely related bacteria, may have close sequence relatives in overall distantly related bacteria (Welch et al., 2002; Ivanova et al., 2003). Thus, ideally, assay development should be coupled to exploration of population genetics and dynamics of the target pathogens and related groups.

2.5.3.1. Hybridization Methods

Fluorescent in situ hybridization. FISH enables detection of specific nucleic acid sequences inside intact cells. Fixed cells are immobilized on microscope slides and permeabilized with chemical reagents. Probes, primarily oligonucleotides (<25 nucleotides long), complementary to specific regions in the cellular DNA or RNA molecules are applied to the cells under optimized incubation and wash conditions. Fluorescent labeling of the probes allows visualization of the target cells by epifluorescent microscopy. Several different labeling techniques are available and include direct labeling of the probe (e.g., FITC or cyanin dye 3) or indirect labeling of probes with enzymes (e.g., horseradish peroxidase), antibodies, or the (strept) avidin system (Moter & Gobel, 2000). For a general review on the use of FISH to detect microbial populations in natural environments see Moter & Gobel (2000).

Several publications have tested the applicability of FISH for environmental detection and enumeration of pathogens or indicators. For example, 16S rRNA targeted oligonucleotide probes have been designed to differentiate Enterobacteriaceae both as a group and as individual species (Loge et al., 1999; Baudart et al., 2002; Rompre et al., 2002). Specifically, *E. coli*, *Enterobacter cloacae*, and *Citrobacter freundii* were identified after membrane filtration followed by FISH for water quality control purposes (Loge et al., 1999; Baudart et al., 2002). This enabled detection of 10^5–10^7 *E. coli* cells per ml of wastewater (Baudart et al., 2002) (Table 2.2). In general, because of the reliance on microscopy, the target population has to be present at >0.1% of the total cell numbers in the community, which translates to $\sim 10^3$ cells per ml for bacterioplankton in most natural waters. However, Colwell and colleagues have combined FISH with the high throughput cell counting ability of flow cytometry and were able to detect *Vibrio* populations at abundances as low as 13 cells per ml (Heidelberg et al., 2002b).

One major problem in FISH arises from the generally low signal level per probe provided by direct labeling procedures, and the low diffusion of large molecules through the cell wall in indirect labeling procedures. This has confined routine FISH application to use of rRNA as targets since these are present in hundreds and thousands of copies in actively growing cells (DeLong et al., 1989; Amann et al., 1990). Unfortunately, the ability of the rRNAs to discriminate among closely related organisms is limited since they are highly conserved molecules and contain only relatively short, variable nucleotide stretches. For example, while it is possible to identify *E. coli* on the species level, pathogenic strains cannot be distinguished

from harmless strains. However, over the past decade several improvements have been made to increase the sensitivity of FISH by use of brighter fluorochromes, signal amplification systems coupled to reporter enzymes, and multiply labeled probes (reviewed in Pernthaler et al., 2002a, 2002b). Thus, it is possible that in the near future more variable targets, such as messenger RNA, will be among the targets for FISH. Furthermore, if flow cytometry can be routinely combined with FISH more efficient sample analysis may arise since labor-intensive microscopy may be circumvented.

Dot-blot and Colony Hybridizations. In all filter hybridizations, nucleic acids are immobilized on membranes and hybridized with specific labeled probes. Various labels are available ranging from radionucleides to biotin or digoxygenin. The latter are detected with antibodies carrying enzymes, which elicit either a color precipitation or chemiluminescent reaction. In dot-blot hybridizations, the target nucleic acids are purified either from isolates or environmental samples; in colony hybridization, filter membranes are applied directly to culture plates and cells are transferred to the membranes, lysed, and their nucleic acids hybridized. In both methods either oligonucleotides against rRNA or polynucleotides against protein-coding mRNA (or genes) can be used as probes and the same considerations of varying ability of different types of probes to discriminate strains and species apply as for all hybridizations (see above).

Although dot-blot hybridization is routinely applied to detect bacterial populations in ecological studies (Koizumi et al., 2002; Polz and Cavanaugh, 1997; Raskin et al., 1994) it has only rarely been applied to monitoring of pathogens. It was recommended for its accuracy, speed, and low cost for detection of drug resistant *M. tuberculosis* strains (Victor et al., 1999), and produced a detection limit of 10^2 cells when albacore tuna muscle extract was artificially contaminated with the pathogen *Stenotrophomonas maltophila* (Ben-Gigirey et al., 2002). Nonetheless, for reliable detection of environmental pathogens, culture enrichment prior to hybridization has been recommended due to uncertain detection limits and possible interference of inhibitors (Straub & Chandler, 2003).

Colony hybridization is essentially an extension of culture-dependent detection of pathogens and, although the same limitations based on culturability apply, it allows rapid, sensitive, and accurate identification of strains. Probes targeting the thermostable direct hemolysin (*tdh*) and/or *tdh*-related hemolysin (*trh*) genes enabled the detection of oyster-associated pathogenic *V. parahaemolyticus* strains at low densities (usually <10 CFU per g of oyster) (Blackstone et al., 2003). Colony hybridization has also been used for the study of the seasonal dynamics of *V. cholerae* along the California coastline with a dynamic range of three orders of magnitude and 1 CFU per ml as the lowest observed abundance (Jiang & Fu, 2001). The USFDA[3] has recognized the high accuracy of colony hybridization and has approved a number of gene targets specific for food- and water-borne pathogens including Listeriolysin O 11 and *msp* genes of *L. monocytogenes*, the invasive genes of *Shigella* spp., enterotoxin B of *S. aureus*, the heat-stable toxin genes of *E. coli*, and the *ail* gene and *inv* genes of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Jackson et al., 2000).

2.5.3.2. Polymerase Chain Reaction

PCR-based detection has revolutionized diagnostic microbiology due to the combination of sensitivity, specificity, and fast turnaround time for identification of infectious agents. The PCR represents an enzymatic copying of specific genes allowing million- to billion-fold

[3] United States Food and Drug Administration.

amplification above the background of single or mixtures of genomes. Double-stranded (genomic) DNA template is denatured and the resulting single strands hybridized with one of two primers, which flank the target gene. These primers are then extended with a thermostable DNA polymerase (e.g., Taq polymerase) generating copies of complementary DNA. This overall process is repeated between 20 and 45 times (cycles) in a single test tube. The specificity of the target amplification is determined by the design of sequence-specific primers and optimization of reaction conditions.

Due to the exponential amplification of templates, the PCR has an exceptional sensitivity of, theoretically, a single target copy; however, the PCR also has high potential for misleading results due to contamination, biases, and inhibition. Contamination most often stems from previous PCR reactions carried out in the same laboratory. For example, aerosols generated by pipetting or opening of reaction tubes are a major source of false positives but use of stuffed tips, laminar flow benches, and UV treatment of tubes can minimize such problems. The PCR is also subject to a number of biases, which generally become more pronounced with increasing reaction cycles. Specifically, after initial exponential amplification, the product accumulation becomes less efficient until a maximum product concentration is reached, which is independent of the amount of starting template. Since the starting template concentration and the efficiency of the amplification are unknown in the reaction, the results of simple PCR assays should never be quantitatively interpreted unless appropriate controls are included (see quantitative PCR (QPCR) below) (von Wintzingerode et al., 1997; Polz & Cavanaugh, 1998). Finally, inhibition of the PCR by environmental contaminants (e.g., humic substances and metal ions) may result in false negatives. To address this problem, various methods have been devised for the purification of nucleic acids from environmental samples prior to amplification (reviewed in von Wintzingerode et al., 1997). It has also been suggested to always include an internal standard in each PCR reaction to indicate possible PCR inhibitors (Malorny et al., 2003).

The unique potential of the PCR for rapid and specific detection of species- or virulence-specific genes has been exploited in numerous assays (for example, reviewed in Straub & Chandler, 2003; Pommepuy & Le Guyader, 1998) (Table 2.2 for detection of pathogens in environmental samples). However, many protocols still utilize some form of enrichment prior to PCR amplification due to the danger of false negatives from inhibition of reaction kinetics by environmental substances. Several techniques have been utilized including filtration, centrifugation, or molecular-based separation (e.g., by magnetic beads). For example, culture-based enrichment increased the sensitivity of a PCR assay for *L. monocytogenes* in household waste samples by several orders of magnitude from 10^7 to 10 CFU per g (Burtscher & Wuertz, 2003). Horgen and colleagues detected *E. coli* at 10 cells per ml of water by concentration of the cells with magnetic beads (Foulds et al., 2002). Other authors used culture enrichment prior to PCR of putative pathogens from water or other environmental samples (Table 2.2).

Several modifications of the PCR technique hold promise for increased accuracy or high-throughput detection of pathogens. The first technique, Quantitative PCR (QPCR), allows quantification of the abundance of target gene sequences in environmental samples. QPCR is available in several formats but real-time QPCR has become the most widely used. It detects the accumulation of DNA template at the end of every cycle. This enables comparison of template accumulation kinetics between environmental samples and standards for accurate quantification (Table 2.2). For higher throughput detection of multiple pathogens in a single test tube, multiplex PCR assays have been developed (Table 2.2). These combine cocktails of specific primers for several targets and allow differentiation of individual amplicons from the mixture of products either by size or labeling of the amplification primers with different fluors.

These examples illustrate just a few of the large number of permutations of the PCR, which have been published. Overall, PCR-based methods are among the most rapid, flexible, and cost effective of the molecular methods, and it is therefore not surprising that many laboratories have concentrated on their use.

2.5.3.3. Molecular Typing Methods

Nucleic-acid-based molecular typing methods allow for the differentiation of strains based on analysis of their genomes. This is important for linking specific strains to disease outbreaks but is also critical for evaluating the specificity of detection methods by providing standards for virulent and harmless strains of the same species. Molecular typing can enable identification of traits unique to virulent strains. Molecular typing methods employed for distinguishing bacterial strains include PFGE, randomly (or arbitrarily) primed PCR, analysis of DNA sequences (e.g., ribosomal genes), and MLST (reviewed in Persing et al., 2003; van Belkum, 2003). Traditionally, the "gold standard" for typing has been PFGE but newer methods such as MLST are rapidly being translated into a format suitable for routine clinical identification of pathogens.

Pulsed Field Gel Electrophoresis. PFGE differentiates genomes by cutting chromosomal DNA with "rare-cutter" restriction enzymes, which due to long recognition sequences cut infrequently. This produces few, large DNA fragments (roughly 10–800 kb), which can be separated by gel electrophoresis under a pulsed-electric field. Both variation in sequence and overall genome architecture are translated into unique patterns of DNA fragments and allow highly specific identification of strains (reviewed in Persing et al., 2003; van Belkum, 2003). PFGE is currently widely applied in food safety assessment and a number of laboratories contribute to the "Foodborne Surveillance PulseNet," a database created by the Centers for Disease Control and Prevention and several state and national laboratories (Binder et al., 1999; Swaminathan et al., 2001). PFGE patterns of strains stored in the database can be compared to those obtained from isolates from contaminated food or clinical samples by electronically submitting images to the network. Standardized PulseNet protocols have been developed for *E. coli* (Breuer et al., 2001), *Campylobacter jejuni* (Ribot et al., 2001), *L. monocytogenes* (Graves & Swaminathan, 2001), and several more are being developed and validated (Swaminathan et al., 2001). Such approaches have high potential for better understanding of the diversity of strains responsible for disease outbreaks; however, PFGE in particular remains a challenging technique to implement reproducibly among different laboratories, and other whole genome comparative methods may ultimately replace PFGE for the routine characterization of isolates.

Multilocus Sequence Typing. One very promising alternative for characterizing bacterial isolates is MLST, which produces nucleotide sequence data that can be readily compared between laboratories and in different studies. In this technique, several defined DNA regions of each bacterial isolate are amplified by PCR and subsequently sequenced. The various sequences of about 500bp are aligned to detect nucleotide differences and sorted into allele homology groups. Since multiple genes are included in the analysis, characteristic allelic profiles can be used to identify pathogenic strains (Maiden et al., 1998; van Belkum, 2003).

2.5.3.4. Future Nucleic Acid-Based Technologies

DNA microarrays hold promise to improve environmental pathogen monitoring by allowing high-throughput detection of multiple pathogen populations in a single analysis. DNA

microarrays allow the differentiation of hundreds to thousands of specific sequences in a sample by simultaneous reverse dot-blot hybridizations [reviewed in (Ye et al., 2001; Call et al., 2003)]. Different, specific probes are attached to a glass slide, and fluorescently labeled target nucleic acids are hybridized to the probes in a single reaction. After stringent washes to remove non-specific hybrids, the hybridization signals are imaged using high-resolution scanners. Recently, DNA microarrays have been applied to the detection of bacteria in soil (Wu et al., 2001) and estuarine water samples (Taroncher- Oldenburg et al., 2003). However, several challenges remain before microarrays can be routinely used for pathogen detection in environmental samples or clinical specimens. Perhaps, the most critical challenge is how to optimize the stringency of the analysis conditions for simultaneous hybridization of multiple probes with different chemical properties. This limitation allows only detection of positive hybridization signals with respect to defined standards and can confound interpretation of hybridization signals from environmental samples due to non-specific cross-hybridization. Furthermore, the cost of equipment, expertise, and large-scale data analysis remains prohibitively high, relegating the use of microarray technology to a few centralized facilities. However, with recent advances in high-throughput genome analysis, microarray technology will prove to be a very valuable tool in clinical and environmental microbiology with applications for the detection and molecular typing of marine pathogens.

The ultimate form of molecular typing is whole genome sequencing. With increased analysis of diverse bacterial genomes, information on strain-to-strain variation and the transfer of virulence properties among bacterial species is becoming available. The genome sequences of over two hundred bacteria have been published, including a number of strains that are marine pathogens, or close relatives thereof (e.g., *Vibrio spp*. including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, *Brucella spp., Clostridium spp*., *Legionella spp., Mycobacterium spp.* and *Shewanella spp.*) and this number is increasing rapidly with advances in high-throughput sequencing technology. Comparative genomic analysis has revealed surprising levels genomic diversity among closely-related bacterial strains (Welch, 2002) and analysis of genomes from pathogenic and non-pathogenic organisms is revealing mechanisms by which pathogenic interactions emerge while providing genetic targets to differentiate virulent from avirulent strains. Such information will be critical for the design of molecular assays to detect and monitor specific pathogens in clinical and environmental settings.

2.6. OUTLOOK

A surprisingly large number of potential human pathogens reside in the marine environment and increased risk of human exposure highlights the need to better understand their ecology and evolution. An integral part of such an effort must be the specific characterization, differentiation, and detection of pathogenic strains. Particular challenges are the potential range expansion of existing marine-indigenous pathogens (e.g., *V. cholerae*) and the emergence of new human-pathogens from marine systems. Indeed, increased reports of disease outbreaks in marine populations may evidence the emergence of new pathogens. The zoonotic potential of such outbreaks in natural or farmed marine environments needs to be recognized and approached with caution while work is done to recognize and prevent the conditions that promote marine disease. The complexity of these problems requires flexible approaches and the overview provided in this chapter attempts to represent methods, which allow both routine

monitoring of pathogens and exploration of their ecology. In the future, coordinated efforts to standardize methods and create databases for comparison will be important for a more comprehensive evaluation of the risk for human populations associated with utilization of marine environments.

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