Potential Aquatic Bacterial Pathogens in the Philippines and Thailand

Studies on the occurrence, characterisation, detection of potential virulence determinants and antimicrobial susceptibility profile

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The present thesis is a partial fulfilment of the requirements for the Master of Science Degree for International Students (MSc) in Veterinary Medicine, at the Swedish University of Agricultural Sciences (SLU), in the field of Veterinary Microbiology

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“When you dream, it is better to aim for the sky…so even if you failed,
you just fall on the roof and not on the floor”

To my family and to the memory of my great mother

ABSTRACT

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Strains of *Aeromonas* spp., `non–cholera vibrios´(NCVs) and *Plesiomonas shigelloides* isolated from aquatic environments and fish and human diarrhoeal cases in the Philippines and Thailand were characterised for potential virulence markers, such as the production of cytotoxin, cell-associated and cell-free haemolysin and their capacity to adhere to human intestinal (Henle 407) cells *in vitro*. In addition, the occurrence of *tlh* and *tdh* haemolysin genes and urease activity among *Vibrio parahaemolyticus* strains were also investigated.

The antimicrobial susceptibility profile of these strains was identified with selected antibiotics used in human medicine and aquaculture. The minimum inhibitory concentration of ampicillin, ceftiofur, gentamicin, neomycin, streptomycin, enrofloxacin, florfenicol, oxytetracycline and trimethoprim-sulphamethoxazole was determined using broth microdilution method. Susceptibility to imipenem and detection of extended-spectrum β-lactamases (ESBLs) in ampicillin resistant strains was achieved by the combination disc diffusion method.

The results showed that strains recovered from clinical sources (human and fish) produced the investigated potential virulence determinants, while these are absent in environmental strains. Such virulence factors may be involved in the pathogenesis of disease caused by these bacterial pathogens in both humans and animals.

Moreover, antimicrobial resistance among environmental strains was rare. Acquired resistance to tetracycline and fluoroquinolone was demonstrated in *Aeromonas* strains of clinical origin and it could be possible that acquired resistance is associated with the use of antimicrobials in therapy.

Key words: Aeromonas *spp.;* Vibrio *spp.;* P. shigelloides*; Philippines; Thailand; virulence determinants; antimicrobial susceptibility*

CONTENTS

INTRODUCTION

Aeromonas spp., `non–cholera vibrios´ (NCVs) and *Plesiomonas shigelloides* belong to an expanding group of food and water-borne pathogens. These bacteria are regarded as important pathogens implicated in both intestinal and extra-intestinal infections in humans and are key pathogens for aquatic animals (Brenden et al., 1988; Pasquale et al., 1994; Martins et al., 2002; Dumontet et al., 2003; Lee et al., 2003). The isolation of these bacteria from fresh and marine water, foods, cold- and warm-blooded animals and humans is well documented (Austin and Austin, 1985; Krovacek et al., 1994a-b; Pascual et al., 1996; Heuzenroeder et al., 1999; Dumontet et al., 2000; Gonzalez-Rey et al., 2003; Martins et al., 2002; DePaola et al., 2003; Lee et al., 2003). Different potential virulence factors of these bacteria have been investigated in various parts of the world. Studies on the production of exotoxins (cytotoxin, cytotonic toxin, enterotoxin, haemolysin) and the ability to bind and invade epithelial cells (Brenden et al., 1988; Carrello et al., 1988; Bartkova and Ciznar 1994; Krovacek et al., 1994a-b; Scoglio et al., 2001; Martins et al., 2002; Falcon et al., 2003) were reported.

Molecular studies have identified the genes responsible for these virulence factors. Virulence genes among *Aeromonas* spp. have been identified (Heuzenroeder et al., 1999; Ormen and Ostensvik 2001; Chacon et al., 2003; Wang et al., 2003). Thermolabile direct haemolysin (*tlh*), thermostable direct haemolysin (*tdh*) and the *tdh*-related haemolysin (*trh*) genes of *V. parahaemolyticus* have also been detected (Okuda et al., 1997; Volety et al., 2001; Kaufman et al., 2002; DePaola et al., 2003).

Susceptibility to antimicrobials differs among *Aeromonas*, *Vibrio* and *P. shigelloides*, although strains are generally susceptible to broad-spectrum cephalosporins, aminoglycosides, quinolones, chloramphenicol and tetracyclines (Kamper et al., 1999; Ottaviani et al., 2001; Alabi and Tolu, 1990). Resistance to these antibiotics has, however, been reported (Goni-Urriza et al., 2000; Marshall et al., 1996).

The occurrence and isolation of these bacteria from different sources has been reported in Asia, including the Philippines and Thailand (Adkins et al., 1987; Ocampo et al., 1989; Haque et al., 1996; Pascual et al., 1996; DePaola et al., 2003). However, in the Philippines information on potential virulence determinants and antibiotic susceptibility of these pathogens, especially those recovered from aquatic environments and aquaculture is scarce.

In this study, the occurrence, characterisation and detection of some potential virulence determinants such as the production of exotoxins (cytotoxin and haemolysin), adhesive properties and haemolysin genes (*tlh, tdh*) was presented along with an antibiotic susceptibility profile of these organisms isolated from different sources in the Philippines and Thailand.

AIMS OF THE INVESTIGATION

1.To determine the occurrence and to characterise and detect potential virulence determinants such as the production of cytotoxins and haemolysins; adhesive properties and presence of haemolysin genes of *Aeromonas* spp., NCVs and *P. shigelloides* isolated from different sources in the Philippines and Thailand.
2. To determine the antimicrobial susceptibility profile of these strains.

STUDY OF LITERATURE

General characteristics of the organisms

Aeromonas spp. belong to the family Aeromonadaceae, *Vibrio* spp. to Vibrionaceae and *P. shigelloides* to Enterobacteriaceae in which members are gram-negative rods (straight or curved), oxidase positive and glucose fermenting. In addition, several species of *Aeromonas* can produce gas, are catalase-positive, facultative anaerobes, and are motile by polar flagella, except *A. salmonicida* which is non-motile. Sodium chloride is needed by several *Vibrio* spp. for growth. *Vibrio* spp. and *Aeromonas* spp. produce several exoenzymes such as DNase, diastase, lipase and various proteinases such as gelatinase. Conversely, *Plesiomonas* does not produce these exoenzymes. Most of the species grow on common laboratory media at 35-37°C; however, many of the saprophytic *Vibrio* and *Aeromonas* species have an optimum temperature for growth lower than 35°C (Quinn et al., 1994; Quinn et al., 2002).

Natural habitat and other sources

Aeromonas spp.

Aeromonas spp. are widely distributed in the environment in both fresh and salt water, sewage and soil (Krovacek et al., 1992; Ivanova et al., 2001; Dumontet et al., 2003). The presence of organic matter in these sources facilitates an increase in number of these organisms (Marcel et al., 2002). Seafood such as shellfish, oysters and fish are common sources of *Aeromonas* (Scoglio et al., 2001). *Aeromonas hydrophila* is part of the normal flora of freshwater fish and is commonly present in fishponds and tanks (Santos et al., 1998; Thayumanavan et al., 2003). *Aeromonas* spp have been recovered from cold-blooded and warm-blooded animals that may serve as faecal carriers of the organisms (Austin and Austin, 1985; Pasquale et al., 1994). In addition, *Aeromonas* spp. have also been isolated from a number of food products of animal origin such as beef, chicken, pork, lamb and raw milk (Krovacek et al., 1992; Martins et al., 2002). Lastly, Khardori and Fainstein (1988) reported the isolation of a small percentage of *Aeromonas* spp. from the stools of healthy individuals.

Non-cholera vibrios (NCVs)

Most NCVs are recovered from salt, brackish and fresh water, but can also be present in the alimentary tracts of animals and man (Dumontet et al., 2000; Ivanova et al., 2001; Pfeffer et al., 2003). Most strains are able to colonise the skin and gastro-intestinal tract of marine vertebrates and invertebrates (Tendencia and Dureza, 1997; Lee et al., 2003).

Plesiomonas shigelloides

Plesiomonas shigelloides is primarily present in aquatic environments and previously thought to be confined to fresh water; however, recent publications have verified isolation from fresh water, brackish and seawater (de Mondino et al., 1995; Aldova et al., 1999; Pasquale and Krovacek, 2001). *Plesiomonas shigelloides* has also been isolated from a wide range of hosts including freshwater fish, shellfish, domestic animals, pets and humans (Arai et al., 1980; Aldova et al., 1999; Bardon, 1999).

Pathogenesis / Clinical infections

Members of these three genera are primarily pathogens of fish and reptiles, although some species can infect mammals and birds (Quinn et al., 2002). Infections are usually opportunistic requiring stress factors for the initiation of disease. The pathogenic mechanisms involved in the causation of disease are not yet fully understood.

Aeromonas spp.

Aeromonas hydrophila causes a disease in fish known as "Motile Aeromonas Septicaemia" (MAS) or Red-Sore Disease", which causes septicaemia and skin ulcers when bacteria or bacterial toxins are present (Swann and White, 1989). *Aeromonas hydrophila, A. caviae, A. veronii* bv *sobria, A. jandaei* and *A. schubertii* are considered as opportunistic human pathogens (Janda et al., 1995; Kirov, 1997). Moreover, *A. hydrophila, A. caviae, A. veronii* bv *sobria* have been suggested as the main cause of *Aeromonas*-mediated human gastroenteritis (Deodhar et al., 1991; Kirov, 1997). *Aeromonas trota* has been isolated from human clinical sources although its role as a causative agent of human disease has not yet been fully elucidated (Janda and Abbott, 1998). *Aeromonas eucrenophila* shows pathogenic potential as demonstrated by fluid accumulation in the rabbit ileal loop test (Singh and Sanyal, 1997). Gastroenteritis caused by *Aeromonas* spp. is self-limiting and usually does not require medical treatment; however extra-intestinal infections may lead to fatal consequences in immunocompromised patients (Krovacek et al., 1993).

Non-cholera vibrios

Non cholera vibrios of clinical importance include *V. cholerae* non-O1, *V. parahaemolyticus, V. vulnificus, V. mimicus, V. fluvialis, V. furnissii, V.* (*Grimontia*) *hollisae, V.* (*Listonella*) *damselae, V. alginolyticus, V. metschnikovii, V. cincinnatiensis and V. harveyi* (*V.charchariae*) (Daniels et al., 2000; Chiang

and Chuang, 2003; Ko et al., 1998; Barber and Swygert, 2000; Hlady and Klonz, 1993). Food poisoning caused by *V. parahaemolyticus* is associated with the consumption of raw or undercooked seafood (Daniels et al., 2000). *Vibrio vulnificus* may cause meningoencephalitis, necrotising wound infections, primary septicaemia and gastroenteritis (Chiang and Chauang, 2003; Kim et al., 2003). *Vibrio metschnikovii* is associated with disease in domestic animals, and causes a cholera-like disease in chickens and other birds although its geographical distribution is limited (Lee et al., 1978). *Vibrio metschnikovii* is also reported from human cases of diarrhoea (Dalsgaard et al., 1996; Magalhaes et al., 1996).

Plesiomonas shigelloides

Plesiomonas shigelloides has been reported as causing gastroenteritis in man, mainly in tropical and subtropical regions (Levy et al., 1998; Albert et al., 1999; Bravo et al., 2000) however, several temperate countries such as Canada (Kain and Kelly, 1989), Finland (Rautelin et al., 1995) and Sweden (Svenungsson et al., 2000) have reported gastrointestinal infections caused by *P. shigelloides*. There are several reports on the role of *P. shigelloides* in extra-intestinal infections, such as bacteraemia, cellulites and meningitis, especially in immunocompromised patients (Clark and Janda, 1991; Jönsson et al., 1998). In addition, *P. shigelloides* has been reported as a potential fish pathogen (Cruz et al., 1986). This bacterium has also been isolated from domestic animals including dogs, cats, goats, sheep and cattle although its role in animal disease is still under investigation (Arai et al., 1980). The most commonly reported species of the three genera that are of humans and veterinary importance are presented in Table 1 (Quinn et al., 1994; Quinn et al., 2002).

The most commonly reported species of the three genera that are of humans and veterinary importance are presented in Table 1 (Quinn et al., 1994; Quinn et al., 2002).

Table 1. *Diseases and hosts of* Aeromonas, Vibrio *and* Plesiomonas *species*

Genus and species	Host(s)	Disease
Aeromonas hydrophila	Amphibians	'Red-leg' syndrome
	Snakes (captive)	Ulcerative stomatitis, Pneumonia, septicaemia
	Freshwater fish	Haemorrhagic septicaemia
	Cattle	Abortion
	Young dogs	Septicaemia
	Humans	Intestinal & Extra-intestinal infections
A. salmonicida	Salmonids	Furunculosis
	Goldfish (carp)	'Ulcer disease'
Vibrio cholerae	Humans	Cholera
V. parahaemolyticus	Humans	Food poisoning associated with seafood
V. metschnikovii	Chickens	Cholera-like enteric disease
V. (*Listonella*) *anguillarum*	Marine fish, eels	Skin lesions, septicaemia
Plesiomonas shigelloides	Fish, reptiles	Septicaemia
	Harbour seals	Diarrhoea
	Humans	Diarrhoea, Neonatal meningitis

Putative virulence determinants

The virulence of an organism refers to its ability to invade and produce disease in a healthy individual or animal. Highly virulent organisms produce serious disease or death in many affected animals, whereas bacteria of low virulence rarely produce serious illness (Quinn et al., 2002). Below is a brief review of some of these virulence determinants:

Enterotoxins

The organisms produce enterotoxins, although the mechanism of action of these enterotoxins is still unclear. Studies have revealed that enterotoxin could inactivate proinflammatory cytokine production and affect metabolism in macrophages, causing tissue damage and a fluid secretory response (Ljungh and Kronevi, 1982; Chopra et al., 2000). In general, enterotoxins are described as either cytotonic enterotoxin or cytotoxic/cytolytic enterotoxin (Keusch & Donta, 1975; Wadström and Ljungh, 1991).

Cytotonic enterotoxins may cause cell-rounding and other morphological changes, without cell death, by stimulation of cyclic-AMP synthesis and steroid secretion by adrenal cells. The toxin's ability to stimulate fluid accumulation in ligated rabbit ileal loop (LRIL) has also been demonstrated (Chakraborty et al., 1984).

Cytotoxic/cytolytic enterotoxins cause severe cell damage and/or death of tissue culture cells and induce accumulation of fluid in LRIL (Keusch & Donta, 1975). Figure 1 demonstrates the cytotoxic effects of bacterial culture filtrates on Vero (African green monkey kidney) cell monolayer. In this case, total or partial destruction of the Vero cells was related to the presence of cytotoxic activity in the samples.

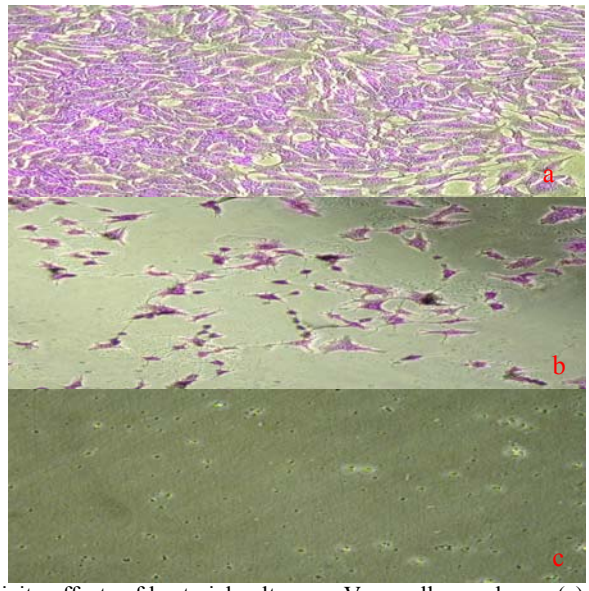

Fig. 1. Cytotoxicity effects of bacterial culture on Vero cell monolayer: (a) normal cell, (b) moderate cell destruction by *A. veronii bv sobria*, (c) total cell destruction and death by *A. hydrophila.*

Haemolysin

This virulence factor is a membrane damaging exotoxin. There are several types of haemolysins, but the most significant is β-haemolysin, which causes complete haemolysis. Haemolysin activity may release components such as haemoglobin, haemin and haematin, which are utilised by the bacteria as a source of iron originating from the host (Daskaleros et al., 1991). Haemolysin may also help release replicated progeny from invaded cells and act as an enterotoxin in the gut (Janda and Abbott, 1993).

Adhesive ability

A vital step for the bacteria to initiate infections is through adherence to host cells, allowing localisation and subsequent colonisation of the appropriate target tissues by the pathogens (Finlay and Falkow, 1997; Scoglio et al., 2001). Figure 2 demonstrates the high adherence of a clinical strain of *A. hydrophila* to human intestinal (Henle 407) cells. One of the most common mechanisms of bacterial adhesion is by fimbriae or pili (Beachey, 1981); other mechanisms may be mediated by non-fimbrial outer membrane proteins (OMP's), bacterial lipopolysaccharide and collagen-binding OMP's (Ascencio et al., 1990; Merino et al., 1996).

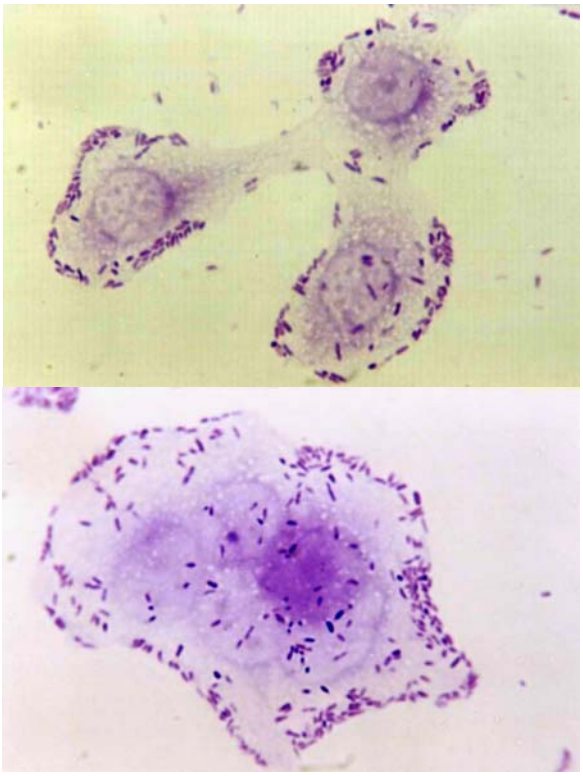

Fig. 2. Aeromonas hydrophila adhesion to Henle 407 cells (Crystal violet stain. Light microscopy X 1250).

Invasive ability

The ability of *Aeromonas* spp., NCVs and *P. shigelloides* to invade epithelial cells has not been fully elucidated and there are few studies on this virulence attribute. (Watson et al., 1985; Theodoropoulos et al., 2001). Merino et al., (1997) demonstrated that the polar flagellum of *A. hydrophila* is essential for the invasion of fish cell lines. In addition, the ability of *Aeromonas* to invade epithelial cells has been associated with dysentery-like diarrhoea (Lawson et al., 1985; Watson et al., 1985). Theodoropoulos et al., (2001) demonstrated that plesiomonads are able to invade human epithelial (Caco-2) cells.

To understand the pathogenic mechanisms of infections caused by *Aeromonas* spp., NCVs and *P. shigelloides*, various studies on virulence factors have been undertaken and these are summarised in Table 2.

Table 2. *Studies on virulence determinants*

Genus	Virulence determinants	Reference
Aeromonas	Enterotoxins	Martins *et al*., 2002
		Singh, 2000
		Trower *et al.*, 2000
		Krovacek *et al*., 1995
	Cytotoxins	Martins *et al*., 2002
		Ormen & Ostenswik, 2001
		Scoglio *et al*., 2001
		Alavandi *et al*., 1999
	Cytotonic toxins	Krovacek *et al*., 1995
		Schulz & Maccardell, 1988
		Chakraborthy *et al*., 1984
		Ljungh *et al*., 1982
	Haemolysins	Thayumanavan *et al*., 2003
		Scoglio *et al*., 2001
		Haque *et al*., 1996
		Santos *et al*., 1998
	Adherence	Gavin *et al*., 2003
		Krovacek *et al*., 1994a
		Ascencio *et al*., 1990
		Carrelo *et al*., 1988
	Invasiveness	Gavin *et al*., 2003
		Merino *et al*., 1997
		Shaw *et al*., 1995
		Krovacek *et al.*, 1995

Table 2. Studies on virulence determinants (Continuation)

Genus	Virulence determinants	Reference
Non-cholera vibrios	Enterotoxins	Singh *et al*., 1996
		Venkateswaran *et al*., 1991
		Datta-Roy *et al*., 1986
	Cytotoxins	Mitra *et al*., 1998
		Wang *et al*., 1998
		Krovacek *et al*., 1994b
		Venkateswaran *et al*., 1991
	Haemolysins	Singh *et al*., 1996
		Venkateswaran *et al*., 1991
		Kelly & Stroh, 1989
		Datta-Roy *et al*., 1986
	Adherence	Wang & Leung, 2000
		Meliotis *et al*., 1995
		Krovacek *et al*., 1994b
		Horne & Baxendale, 1983
	Invasiveness	Meliotis *et al*., 1995
		Shinoda *et al*., 1985
		Smith & Merkel, 1982
P. shigelloides	Enterotoxins	Falcon *et al*., 2003
		Abbott *et al*., 1991
		Matthews *et al*., 1988
	Cytotoxins	Ekman 2003
		Falcon *et al*., 2003
		Abbott *et al*., 1991
		Olsvik *et al*., 1990
	Haemolysins	Falcon *et al*., 2003
		Baratela *et al*., 2001
		Santos *et al*., 1999
		Janda& Abbott, 1993
	Adherence	Ekman, 2003
		Schubert & Holz-Bremer (1999)
	Invasiveness	Theodoropoulos *et al*., 2001
		Olsvik *et al*., 1985
		Binns *et al*., 1984

The role of exoenzymes, including amylase, DNAse, RNAse, esterase, lipase, gelatinase, protease, chitinase, mucinase and hyaluronidase, among others, in the virulence of *Aeromonas* spp. and NCVs have also been investigated (Desmond et al., 1984; Janda, 1985; Oliver et al., 1986; Krovacek et al., 1994b; Majeed, 1996).

In recent years, molecular studies have also been performed to identify the genes responsible for these virulence factors. Such studies include the identification of virulence genes among *Aeromonas* spp. (Heuzenroeder et al., 1999; Ormen and Ostensvik 2001; Chacon et al., 2003; Wang et al., 2003) and the detection of

thermolabile direct haemolysin (*tlh*), thermostable direct haemolysin (*tdh*) and the *tdh*-related haemolysin (*trh*) genes of *V. parahaemolyticus* (Okuda et al., 1997; Volety et al., 2001; Kaufman et al., 2002; DePaola et al., 2003).

Genotyping

Molecular studies are increasingly used for genotyping of bacteria and are specifically useful for epidemiological purposes. Intra-specific typing of strains is vital for the recognition of disease outbreaks, the determination of infection source, the detection of particularly virulent strains, as well as the study of the geographical and host distribution of possible variants of a specific pathogen (Olive & Bean, 1999).

Molecular techniques, including ribotyping (RT), pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consencus (ERIC-PCR), repetitive extragenic palindromic-PCR (REP-PCR) and random amplified polymorphic DNA (RAPD), have been already used for *Aeromonas* spp., NCVs and *P. shigelloides* (Shigematsu et al., 2000; Blackstone et al., 2003; Borchardt et al., 2003; Ripabelli et al., 2003; Villari et al., 2003; Szczuka & Kaznowski, 2004).

Antibiotic susceptibility profile

Antibiotic susceptibility profiles differ among the species in the three genera, but strains are generally resistant to ampicillin, piperacillin, carbenicllin and other penicillin, and are susceptible to broad-spectrum cephalosporins, aminoglycosides, quinolones, chloramphenicol and tetracyclines.

Aeromonas spp.

Aeromonas spp. are reported to be susceptible *in vitro* to broad-spectrum cephalosporins, aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulphamethoxazole (Koehler and Ashdown, 1993; Motyl et al., 1985), aztreonam and fluoroquinolones (Ko et al., 1996). However, an increasing resistance to broad-spectrum cephalosporins in clinical *Aeromonas* strains has been reported (Ko et al., 1996). Most *Aeromonas* spp., including *A. hydrophila, A. caviae, A. veronii* bv *veronii and A. eucrenophila* are inherently resistant to ampicillin (Vila et al., 2002; Abbott et al., 2003). *Aeromonas media, A. sobria, A. salmonicida* and *A. trota* have varying ampicillin susceptibility, with *A. trota* reported as inherently susceptible to ampicillin (Carnahan et al., 1991; Overman and Janda, 1999; Vila et al., 2002; Abbott et al., 2003).

Non-cholera vibrios

Antibiotic susceptibilities differ among species, but strains are generally susceptible to chloramphenicol, tetracycline, aminoglycosides, quinolones and β-lactams; however, multiple antibiotic-resistant strains were reported (Dalsgaard et al., 1999; Roque et al., 2001; Molina-Aja et al., 2002). Most strains are inherently resistant to ampicillin and cases of β-lactam resistance are now widespread among vibrios from different sources (Hasegawa et al., 1986; Molina-Aja et al., 2002).

Plesiomonas shigelloides

Plesiomonas shigelloides is susceptible to second and third generation cephalosporins, carbapenems, aztreonam, trimethoprim-sulphamethoxazole, chloramphenicol, quinolones, fosfomycin, nitrofurantoin, nalidixic acid and co-trimoxazole (Stock and Wiedemann, 2001a; Stock and Wiedemann, 2001b; Gonzalez-Rey, et al., 2004). However, occurrences of strains resistant to chloramphenicol and co-trimoxazole have been reported (Wong et al., 2000). *Plesiomonas shigelloides* is naturally resistant to ampicillin and is inherently resistant and intermediate to streptomycin, erythromycin and rifampin. Some strains may also carry resistant genes to tetracycline (Stock and Wiedemann, 2001b; Gonzalez-Rey, et al., 2004).

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RESEARCH REPORT 1

OCCURRENCE, CHARACTERISATION AND DETECTION OF POTENTIAL VIRULENCE DETERMINANTS OF AQUATIC BACTERIAL PATHOGENS FROM THE PHILIPPINES AND THAILAND

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SUMMARY

Strains of *Aeromonas* spp., `non–cholera vibrios´(NCVs) and *Plesiomonas shigelloides* isolated from aquatic environments, fish and human diarrhoeal cases in the Philippines and Thailand were characterised for potential virulence markers, such as the production of cytotoxin, cell-associated and cell-free haemolysin and their capacity to adhere to human intestinal (Henle 407) cells *in vitro*. In addition, the occurrence of *tlh* and *tdh* haemolysin genes and urease activity among *V. parahaemolyticus* strains were investigated. The results showed that strains recovered from clinical sources (human and fish) produced these virulence factors, whereas these are absent in environmental strains. These factors may be involved in the pathogenesis of disease caused by these bacterial pathogens in both humans and animals.

KEY WORDS: *Aeromonas spp.; Vibrio spp.; P. shigelloides; Philippines; Thailand; virulence determinants*

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INTRODUCTION

Aeromonas spp., `non–cholera vibrios´ (NCVs) and *Plesiomonas shigelloides* belong to the expanding group of food and water-borne pathogens. These bacteria are regarded as important pathogens implicated in both intestinal and extra-intestinal infections in humans and are important pathogens of aquatic animals (Brenden et al., 1988; Pasquale et al., 1994; Martins et al., 2002; Lee et al., 2003). The isolation of these bacteria from fresh and marine water, foods, cold- and warm-blooded animals and humans is well documented (Austin and Austin, 1985; Krovacek et al., 1994a,b; Pascual et al., 1996; Heuzenroeder et al., 1999; Dumontet et al., 2000; Gonzalez-Rey et al., 2003; Martins et al., 2002; DePaola et al., 2003; Dumontet et al., 2003; Lee et al., 2003).

Different potential virulence factors of these bacteria have been studied globally. Thus, the production of exotoxins (cytotoxin, cytotonic toxin, enterotoxin, haemolysin) and the ability to bind and invade epithelial cells have been reported (Brenden et al., 1988; Carello et al., 1988; Bartkova and Ciznar 1994; Krovacek et al., 1994a,b; Scoglio et al., 2001; Martins et al., 2002; Falcon et al., 2003). Molecular studies have also identified the genes responsible for these virulence factors, including the identification of virulence genes among *Aeromonas* spp. (Heuzenroeder et al., 1999; Ormen and Ostensvik 2001; Chacon et al., 2003; Wang et al., 2003). The thermolabile direct haemolysin (*tlh*), thermostable direct haemolysin (*tdh*) and the *tdh*-related haemolysin (*trh*) genes of *V. parahaemolyticus* have also been detected (Okuda et al., 1997; Volety et al., 2001; Kaufman et al., 2002; DePaola et al., 2003b).

The occurrence and isolation of these bacteria from different sources have also been reported in Asia, including the Philippines and Thailand (Adkins et al., 1987; Ocampo et al., 1989; Haque et al., 1996; Pascual et al., 1996; DePaola et al., 2003b). However, only limited information on these aquatic pathogens is available, particularly from the Philippines.

The aim of the present study was to determine the occurrence; and to characterise and detect some potential virulence determinants of these bacteria from the Philippines and Thailand, such as the production of exotoxins (cytotoxin and haemolysin) and adhesive properties. In addition, the presence of haemolysin genes (*tlh, tdh*) and urease among *V. parahaemolyticus* were also investigated.

MATERIALS AND METHODS

Identification, characterisation and occurrence of bacterial isolates

A total of 38 isolates were recovered from aquaculture and aquatic environments in the Philippines (n=27) and from organs of diseased fishes and diarrhoeal human patients in Thailand (n=11). Isolates, which were gram-negative rods, oxidase positive and glucose fermenting were further characterised with API 20E (bio Merieux, Marcy-l'Etoile, France). Isolates identified as *Aeromonas* spp. were further classified by the Aerokey II scheme of Carnahan et al., (1991) and with the phenotypic identification scheme of Abbott et al., (2003). Further identification

and verification was carried-out at the Microbial Diseases Laboratory, California Department of Health Services, USA. *Vibrio parahaemolyticus* strains were confirmed at the Gulf Coast Seafood Laboratory, Food and Drug Administration, Alabama, USA by *tlh* gene probe (McCarthy et al., 1999); while the *V. cholerae* strains were confirmed with PCR and serotyped at the Swedish Institute for Infectious Disease Control, Solna, Sweden. *Plesiomonas shigelloides* strains were confirmed and serotyped (at the Institute of Preventive and Clinical Medicine, Bratislava, Slovak Republic) according to the scheme of Shimada and Sakazaki (1978) and by the International Antigenic scheme as described by Aldova and Schubert (1996).

Growth temperature requirement

Potential virulence factor production was determined on all strains after incubation at 28°C for 48 h and 37°C for 24.

Culture condition for detection of potential virulence factors

The preparation of bacterial culture filtrates was conducted after the work of Falcon et al., (2003). Each strain of bacteria was cultured in 10 ml of Brain Heart Infusion (BHI) broth (Oxoid, Hampshire, England) at 28°C for 24 h and 37°C for 18 h, shaken at 110 rev/min with turbidity reaching Mc Farland #4 standard (bio Merieux, Marcy-l'Etoile, France). Bacterial cultures were centrifuged at 10 000 g at 28°C for 10 min. The sterile culture supernatant was filtered through 0.22 µm filtres (Millipore, Bedford, MA, USA) and collected individually into 15 ml plastic test tubes (Greiner bio-one, Kremsmenster, Austria). Sterile cell-free culture filtrates were tested for presence of cytotoxin and haemolysin as described below.

Cytotoxicity assays

The Vero (African green monkey kidney) cell-line, obtained from the Department of Virology, National Veterinary Institute (SVA), Uppsala, Sweden, was used for the cytotoxicity assay. The assay was conducted according to the method of Falcon et al., (2003). The cell-line was grown for 3-5 days in a tissue culture flask with Eagle's Minimum Essential Medium (MEM; SVA, Uppsala, Sweden) supplemented with 7% (v/v) foetal calf serum, 50 units of Penicillin G and 50 µg of Streptomycin/ml. Monolayer cells were detached from the flask with Phosphate Buffer-Trypsin (SVA, Uppsala, Sweden), resuspended to ca. 10^4 cells/ml in MEM. A volume of 200 µL of the resuspended cells was pipetted into a 96-well microtitre plate and incubated at 36°C in 5% CO_2 for 24 h. Confluent monolayers were exposed to different dilutions (1:1, 1:10 and 1:100) of the sterile bacterial culture filtrates. *Aeromonas hydrophila* strain ATCC 15467 was used as a positive control and sterile normal physiological saline solution served as a negative control. The plates were then incubated at 37°C in 5% CO_2 for 24 h. After incubation, the plates were fixed with absolute methanol for 10 min, rinsed with tap water and stained with 2% crystal violet for 5 min. The cytotoxic effect of the bacterial culture filtrates was observed with an inverted microscope (Nikon Eclipse TS100, Japan) with an in-built digital camera (Nikon Coolpix 4500,

Japan). Total or partial destruction of the Vero cells was related to the presence of cytotoxic activity in the samples.

Detection of haemolytic activity

Detection of cell-associated haemolysin

Strains were streaked onto Blood Agar Plates (BAP) composed of tryptone soya agar (Oxoid, Hampshire, England) containing 5% (v/v) bovine erythrocytes. The plates were incubated at 28°C for 48 h and 37°C for 24 h. Complete haemolysis around the colonies was identified as ß-haemolysis.

Detection of cell-free haemolysin

Five wells of ca. 6 mm in diameter were punched into the BAP. The bacterial culture filtrate was then pipetted into these wells and incubated at 28°C for 48 h and 37°C for 24 h. Complete haemolysis around the punched wells was identified as ß-haemolysis.

Thermostability assays

The sterile bacterial culture filtrates were heated in a water bath at 56°C for 15 min and at 80°C for 10 min, then cooled rapidly and tested for cytotoxicity and haemolytic activities as described above.

In vitro *adhesion to human intestinal (Henle 407) cells*

Aeromonas hydrophila and *Vibrio* spp. strains were tested for their ability to adhere to human intestinal (Henle 407) cells *in vitro* (Department of Virology, SVA, Uppsala, Sweden). The procedure was conducted after the work of Krovacek et al., (1987). The Henle 407 cells were seeded to grow in monolayers on glass slides dipped in tissue culture medium (Eagle's Minimum Essential Medium (MEM), SVA, Uppsala, Sweden) containing 10% foetal calf serum, 50 units of Penicillin G and 50 µg of Streptomycin/ml. In sterile petri dishes, the tissue culture cells were then incubated with bacterial suspension (10^6-10^8 bacteria /ml) for 2 h and were continuously shaken gently. The slides were then washed thoroughly to eliminate non-attached bacteria by dipping several times in a series of beakers, each containing 400 ml 0.145 M NaCl. The washed slides were fixed by absolute methanol and stained with 2% crystal violet for 5 min. Finally, the slides were examined under a light microscope (Leitz Larorlux K, Germany) and the average number of bacteria adhering per Henle 407 cell was determined under 1250x magnification.

Determination of tlh *and* tdh *genes in* V. parahaemolyticus *strains*

Preparation of *V. parahaemolyticus* strains for *tlh* and *tdh* genes determination was patterned after the methods described by McCarthy et al., (1999 and 2000). The strains were inoculated on T1N3 agar and incubated at 28°C for 24 h. Colony lifts were made by placing an 85 mm Whatman #541 filtre (Maidstone, England) on the surface of each T1N3 plate with colonies. The filtre paper was then removed and placed (colony side-up) in an inverted 100x15 mm glass petri dish lid with 1

ml lysis solution. The filter paper was placed inside a microwave and heated on full power (100 watts) for 30 sec/filter. The filter paper was then washed with 4 ml of ammonium acetate buffer for 5 min at 28°C with continuous swirling in an orbital shaker. The ammonium acetate buffer was decanted and 10 ml of 1x Standard Saline Citrate (SSC) solution per filter was added and let to stand for 1-2 min with continuous swirling. The liquid was decanted and the filtre paper was rinsed again with 1x SSC solution. The filter papers were allowed to air dry at room temperature. The filtres were then sent to the Gulf Coast Seafood Laboratory, FDA, Dauphin Island, Alabama, USA for the detection of the *tlh* and *tdh* genes by DNA gene probes as previously described (McCarthy et al., 1999 and 2000).

Urease activity in V. parahaemolyticus *strains*

The *V. parahaemolyticus* strains were also tested for urease activity by inoculation on urea broth (Oxoid, Hampshire, England) and incubation at 28°C for 48 h. Positive reaction was indicated by a change in medium colour to dark pink.

RESULTS

Identification, characterisation and occurrence of bacterial isolates

Among the 38 isolates studied, eight were identified as *Aeromonas hydrophila*; one as *A. veronii* bv *sobria*; 11 as *Vibrio* spp (non-human, environmental); 14 as *V. parahaemolyticus*; two as *V. cholerae* Non O-1, non O139; and two as *Plesiomonas shigelloides* (serotypes O77:H22 and NA:H21) The distribution, sources and country of origin of these strains are listed in Table 1.

Growth temperature requirement

Among the 38 strains, nine strains only grew at 28°C. All of these were recovered from aquatic environments and were identified as *Vibrio* spp that are non-pathogenic for humans. The remaining 29 strains both grew at 28°C and 37°C (Table 2).

Cytotoxicity Assays

Table 2 summarises the results for cytotoxicity assay. All strains (n=9) recovered from human diarrhoeal patients and diseased fishes were cytotoxin producers and comprised of seven strains of *A. hydrophila*; one *A. veronii* bv *sobria*, and one *V. cholerae* Non O-1. Conversely, strains from environmental sources showed no cytotoxic reaction in the cell test, except for one strain of *V. cholerae* Non O-1. The highest cytotoxin titre (1:100) was observed in *Aeromonas* spp. strains incubated at 28°C.

The cytotoxic activities were lost after the culture filtrates were heated at 56°C for 15 min and 80°C for 10 min, except for one strain of *A. veronii* bv *sobria*, isolated from diseased catfish: this strain demonstrated weak cytotoxic activity using undiluted filtrate (1:1) after exposure at 56°C for 15 min (Figure 1).

Detection of haemolytic activity

Ten out of 38 strains investigated displayed ß-haemolysis on BAP at both 28°C and 37°C incubation. Of these ten strains, eight strains displayed both cell-associated and cell-free haemolysis whereas the remaining two strains only displayed cell-associated haemolysis. The ß-haemolysis producing strains were recovered from human diarrhoeal patients and diseased fish. Among the strains of *Aeromonas*, all clinical strains of *A. hydrophila* (n=7) and *A. veronii* bv *sobria* (n=1) displayed ß-haemolysis on BAP. Among the NCVs, both strains of *V. cholerae* Non O-1 were ß-haemolytic. None of the *Vibrio* spp. and *V. parahaemolyticus* were β-haemolytic. Both strains of *P. shigelloides* were also non-haemolytic (Table 2). The haemolytic activity was inhibited after exposing the culture supernatant at 56°C for 15 min and at 80°C for 10 min.

In vitro *adhesion to human intestinal (Henle 407) cells*

The results for *in vitro* adhesion to Henle 407 cells are summarised in Table 3. All *A. hydrophila* strains (n=6) from clinical sources adhered to human intestinal (Henle 407) cells with $\geq$30 bacteria per Henle 407 cell (Figure 2). Interestingly, one environmental strain of *A. hydrophila* did not demonstrate any adherence. In addition, all the *Vibrio* spp. (non-human, environmental) also did not exhibit any adherence to Henle 407 cells.

Determination of tlh *and* tdh *genes and urease activity in* V. parahaemolyticus s*trains*

All investigated strains of *V. parahaemolyticus* (n=14) were *tlh* positive, *tdh* and urease negative in all strains.

DISCUSSION

Infections caused by *Aeromonas* spp., NCVs, and *P. shigelloides* in humans and animals have consequently generated worldwide interest in recent years. Since these pathogens are widely distributed in the environment, particularly in aquatic systems, it is thus possible that such environments are important reservoirs for these bacteria. In the present study, we tried to determine if some of these potential virulence determinants are present in both environmental and human strains.

In a report from Japan, incidence of various enteropathogenic bacteria was examined from faecal samples collected from diarrhoeal patients in Kobe City and from overseas traveller covering the period 1989-1999 (Murase et al., 2001). The results from this study showed that the members of the genus *Vibrio*, *Aeromonas* and *Plesiomonas* were abundant from domestic and overseas samples. Moreover, *V. parahaemolyticus* and *P. shigelloides* were reported among the most frequently isolated species. In another study, *P. shigelloides* ranks first in the group of enteropathogens that were recovered from faecal samples of Japanese patients with traveller's diarrhoea (Ueda et al., 1999).
Many strains of *Aeromonas* spp., NCVs and *P. shigelloides* posses different potential virulence markers including invasivity and adherence to eukaryotic cells,

production of cytotoxins, cytotonic toxins, enterotoxins and haemolysins (Brenden et al., 1988; Carello et al., 1988; Bartkova and Ciznar 1994; Krovacek et al., 1994a,b; Scoglio et al., 2001; Martins et al., 2002; Falcon et al., 2003). In this study, strains that produced both cytotoxins and haemolysins came from diseased fishes and human diarrhoeal patients, except for one strain of *V. cholerae* Non-O1 isolated from source/creek water. Except for the latter strain, none of the environmental strains was cytotoxic or haemolytic. Production of these exototoxins is reported in several studies (Krovacek et al., 1994a; Ormen and Ostensvik, 2001; Scoglio et al., 2001; Martins et al. 2002). Kaper et al., (1981) studied 118 *Aeromonas* isolates from Chesapeake Bay, USA, of which 71% were cytotoxin producers and 73% were enterotoxin producers. In Australia, Burke et al., (1984) determined that 70% of *Aeromonas* spp. isolated from water was enterotoxin positive. In a report from Italy, Scoglio et al., (2001) identified *A. hydrophila* strains recovered from raw seafood products as cytotoxin producers.

In this study, the production of cell-associated haemolysis was higher than the production of cell-free haemolysis, which supports the findings of Martins et al., (2002), who suggested that this difference is due to different types of genes being responsible for each type of haemolysis.

Both investigated strains of *P. shigelloides* were non-cytotoxic and non-haemolytic. However a report from Falcon et al., (2003), demonstrated the vacuolation effect of four *P. shigelloides* strains on Vero cell monolayer and their haemolytic activities. In addition, Ekman (2003) reported some cytotoxic effects on Vero cell monolayer by *P. shigelloides* strains recovered from humans, animals and aquatic environments. On the other hand, Vitovec et al., (2001) did not demonstrate any cytotoxic and haemolytic effect by *P. shigelloides* strain in experimental mono-and coinfection with *Cryptosporidium parvum*.

The present study also determined that eight strains of *Vibrio* spp. (non-human, environmental) did not grow at 37°C. Production of cytotoxin and haemolysin was more pronounced when the strains were incubated at 28°C rather than at 37°C. For instance, strains of *A. hydrophila* produced higher cytotoxin titre when incubated at 28°C. These results support the findings of Krovacek et al., (1991), Majeed and Mac Rae (1991), Hudson and Avery (1994) and Majeed (1996), who all reported that *Aeromonas* spp. have an optimal temperature of below 37°C and can even survive at temperatures as low as 4°C.

The results of heat inactivation of the sterile culture filtrates revealed that all positive strains (n=10) lost their cell-free ß-haemolytic property, and a majority (n=9) lost their cytotoxic activity on Vero cell monolayer when heated at 56°C for 15 min and at 80°C for 10min. Other studies have also reported that these exotoxins are heat labile (Baratela et al., 2001; Falcon et al., 2003).

A correlation between haemolysis and cytotoxin production was demonstrated in the present study. Heuzenroeder et al., (1999) and Wang et al., (2003) demonstrated that the presence of haemolysis genes is related to the production of cytotoxin.

The ability to bind to intestinal cells is a first step in the colonization and development of disease for many enteropathogenic bacteria. Interestingly, all investigated strains of *A. hydrophila* from human diarrhoeal patients and diseased fishes adhered to human intestinal (Henle 407) cells *in vitro* (Table 3). On the other hand, all environmental strains including one *A. hydrophila* and 11 *Vibrio* spp. did not demonstrate any adherence. This result supports the findings of Carrello et al., (1988) who determined that clinical isolates of *Aeromonas* spp. were highly adhesive to Hep-2 (human carcinoma larynx) cells, whereas, isolates from water samples had low adherence. Moreover, Krovacek et al., (1994a) reported that clinical strains of *A. hydrophila* demonstrated stronger adherence ability to human intestinal cells than the environmental strains.

The occurrence of haemolysis genes (*tlh* and *tdh*) and urease activity were determined among *V. parahaemolyticus* strains. The *tlh* gene is unique for *V. parahaemolyticus* and thus the presence of this gene is confirmatory for this species (McCarthy et al., 1999). In the present study, all 14 strains of *V. parahaemolyticus* were positive for this gene. The *tdh* gene is considered as a major virulence gene of *V. parahaemolyticus* and is found in a low percentage (<1%) of food and environmental isolates but in over 90% of clinical isolates (DePaola et al., 2003a). Molecular studies lead to support a correlation among *tdh* and the presence of *trh* gene and urease activity. Such studies also indicated that some isolates coming from cases of gastroenteritis are *tdh/trh* and urease positive (Okuda et al., 1997; Kaufman et al., 2002; DePaola et al., 2003). In the present study, the *tdh* gene was absent as well as the production of urease. Unpublished data using an experimental DNA probe for colony hybridisation did not detect *trh* gene in any of the *V. parahaemolyticus* strains examined in the present study.

In conclusions, this study highlighted the strong association of virulence factors in clinical strains recovered from human diarrhoeal patients and diseased fishes whereas lacking in environmental strains. These potential virulence determinants may be involved in the pathogenesis of the disease in both humans and animals.

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Table 1. *Distribution and sources of* Aeromonas *spp., NCVs and* P. shigelloides.

No.	Species	Country	Source
1	*A. hydrophila*	Thailand	human patient
4	*A. hydrophila*	Thailand	human patient
5	*A. hydrophila*	Thailand	human patient
6	*A. hydrophila*	Thailand	human patient
7	*A. hydrophila*	Thailand	human patient
8	*A. hydrophila*	Thailand	human patient
13	*A. hydrophila*	Thailand	nile tilapia (*Oreochromis nilotica*) liver
17	*A. hydrophila*	Philippines	source/creek water
10	*A. veronii* bv. *sobria*	Thailand	spotted ghost catfish(*Ompok bimaculatus*) liver
34	*Vibrio* spp.	Philippines	pond sediment
47	*Vibrio* spp.	Philippines	reservoir water
49	*Vibrio* spp.	Philippines	reservoir water
58	*Vibrio* spp.	Philippines	pond water
61	*Vibrio* spp.	Philippines	reservoir water
68	*Vibrio* spp.	Philippines	reservoir water
69	*Vibrio* spp.	Philippines	source water
70	*Vibrio* spp.	Philippines	source water
71	*Vibrio* spp.	Philippines	source water
72	*Vibrio* spp.	Philippines	source water
75	*Vibrio* spp.	Philippines	rearing water of *Penaeus monodon*
16	*V. parahaemolyticus*	Philippines	source/creek water
18	*V. parahaemolyticus*	Philippines	pond water
19	*V. parahaemolyticus*	Philippines	reservoir water
41	*V. parahaemolyticus*	Philippines	Crab haemolymph
44	*V. parahaemolyticus*	Philippines	pond water
51	*V. parahaemolyticus*	Philippines	source/creek water
54	*V. parahaemolyticus*	Philippines	source/creek water
67	*V. parahaemolyticus*	Philippines	source/creek water
77	*V. parahaemolyticus*	Philippines	rearing water of *P.monodon*
90	*V. parahaemolyticus*	Philippines	*P. monodon*
92	*V. parahaemolyticus*	Philippines	*P. monodon*
96	*V. parahaemolyticus*	Philippines	Crab haemolymph
99	*V. parahaemolyticus*	Philippines	*P. monodon*
100	*V. parahaemolyticus*	Philippines	*P. monodon*
12	*V. cholerae* Non-O1	Thailand	hybrid catfish (*Clarias macrocephalus*) liver
15	*V. cholerae* Non-O1	Philippines	source/creek water
11	*P. shigelloides* O77:H22	Thailand	hybrid catfish (*Clarias macrocephalus*) kidney
14	*P. shigelloides* NA:H22	Thailand	striped catfish (*Pangasius sutchi*) liver

TABLE 2. *Potential virulence determinants of aquatic bacterial pathogens examined*

		Cytotoxin[1]		Haemolysin Production			
				Cell-Associated (BAP)		Cell-Free	
No.	Species	28^0C^2	37^0C^2	28^0C	37^0C	28^0C^2	37^0C^2
1	*A. hydrophila*	10	1	+	+	+	+
4	*A. hydrophila*	1	1	+	+	-	-
5	*A. hydrophila*	10	10	+	+	+	+
6	*A. hydrophila*	1	1	+	+	-	-
7	*A. hydrophila*	100	10	+	+	+	+
8	*A. hydrophila*	100	10	+	+	+	+
13	*A. hydrophila*	10	10	+	+	+	+
17	*A. hydrophila*	-	NG	-	NG	-	NG
10	*A. veronii* bv *sobria*	100	100	+	+	+	+
34	*Vibrio* spp.[3]	-	NG	-	NG	-	NG
47	*Vibrio* spp.	-	NG	-	NG	-	NG
49	*Vibrio* spp.	-	NG	-	NG	-	NG
58	*Vibrio* spp.	-	-	-	-	-	-
61	*Vibrio* spp.	-	NG	-	NG	-	NG
68	*Vibrio* spp.	-	-	-	-	-	-
69	*Vibrio* spp.	-	NG	-	NG	-	NG
70	*Vibrio* spp.	-	NG	-	NG	-	NG
71	*Vibrio* spp.	-	NG	-	NG	-	NG
72	*Vibrio* spp.	-	NG	-	NG	-	NG
75	*Vibrio* spp.	-	NG	-	NG	-	NG

[1] Titres represent the reciprocal of highest dilution of supernatants resulting in cytotoxic effect on at least 50% of the Vero cells.

[2] Incubation temperature of BAP

[3] Species that are pathogenic for humans were ruled out using 25 biochemical tests that include the following species: *V. cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus, V. fluvialis, V. furnissii, V.* (*Grimontia*) *hollisae, V.* (*Listonella*) *damselae, V. alginolyticus, V. metschnikovii, V. cincinnatiensis, V. harveyi* (*V.charchariae*)

NG = No growth

TABLE 2. *Potential virulence determinants of aquatic bacterial pathogens examined* (Continuation)

		Cytotoxin[1]		Haemolysin Production				*tlh*[2]	*tdh*[2]
				Cell-Associated (BAP)		Cell-Free			
No.	Species	28^0C[3]	37^0C[3]	28^0C	37^0C	28^0C[3]	37^0C[3]		
16	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
18	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
19	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
41	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
44	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
51	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
54	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
67	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
77	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
90	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
92	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
96	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
99	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
100	*V. parahaemolyticus*	-	-	-	-	-	-	+	-
12	*V. cholerae* Non-O1	1	1	+	+	+	+		
15	*V. cholerae* Non-O1	1	10	+	+	+	+		
11	*P. shigelloides* O77:H22	-	-	-	-	-	-		
14	*P. shigelloides* NA:H22	-	-	-	-	-	-		

[1] Titres represent the reciprocal of highest dilution of supernatants resulting in cytotoxic effect on at least 50% of the Vero cells.

[2] Only *V. parahaemolyticus* strains were tested.

[3] Incubation temperature of BAP

NG = No growth

Note: Unpublished data using an experimental DNA probe for colony hybridization did not detect *trh* in any of the *V. parahaemolyticus* strains used in the present study

TABLE 3. *Adhesion to human intestinal (Henle 407) cells* in vitro

No.	Species	Source	Adhesion[1]
1	*A. hydrophila*	human patient	≥30
4	*A. hydrophila*	human patient	≥30
5	*A. hydrophila*	human patient	≥30
6	*A. hydrophila*	human patient	≥30
7	*A. hydrophila*	human patient	≥30
8	*A. hydrophila*	human patient	≥30
17	*A. hydrophila*	source/creek water	-
34	*Vibrio* spp.	pond sediment	-
47	*Vibrio* spp.	reservoir water	-
58	*Vibrio* spp.	pond water	-
68	*Vibrio* spp.	reservoir water	-
69	*Vibrio* spp.	source water	-
70	*Vibrio* spp.	source water	-
71	*Vibrio* spp.	source water	-
72	*Vibrio* spp.	source water	-

[1] Average number of bacterial cell adhering/Henle 407 cells

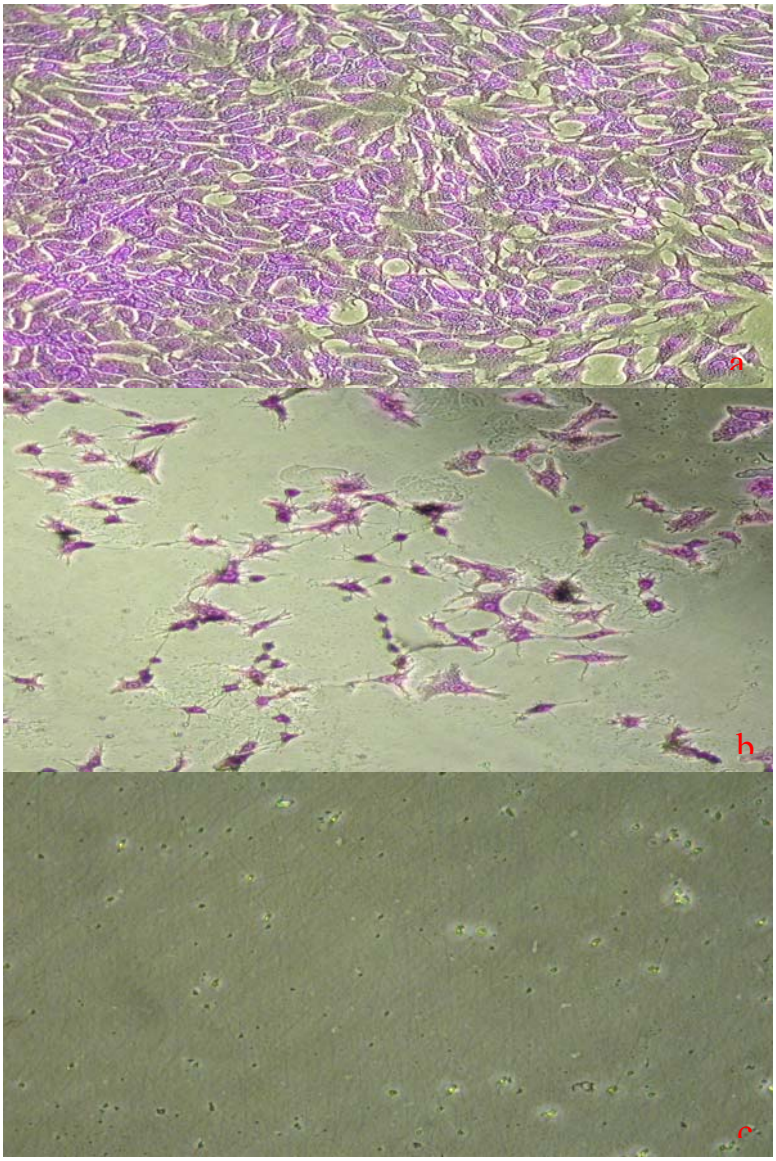

Fig. 1. Cytotoxicity effects of bacterial culture filtrates on Vero cell monolayer: (a) normal cell, (b) moderate cell destruction by *A. veronii* bv *sobria,* (c) total cell destruction and cell death by *A. hydrophila.*

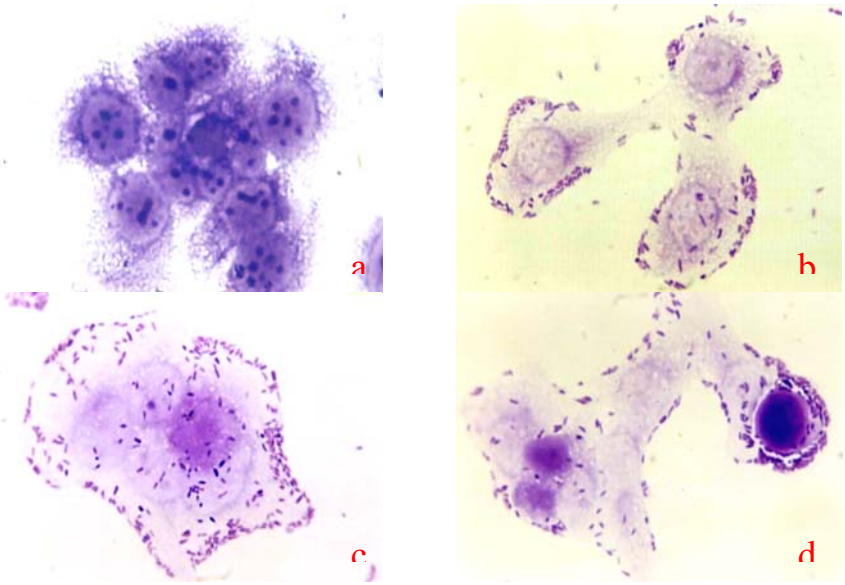

Fig. 2. Bacterial adhesion to Henle 407 cells: (a) normal cell, (b-d) high adherence of *A. hydrophila* strains at ≥30 bacterial cell /Henle 407 cell

RESEARCH REPORT 2

Antimicrobial susceptibility of *Aeromonas* spp., *Vibrio* spp. and *Plesiomonas shigelloides* isolated in the Philippines and Thailand

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Abstract

The susceptibility of 38 strains of *Aeromonas* spp., non-cholera vibrios (NCVs) and *Plesiomonas shigelloides* to selected antimicrobials used in human medicine and aquaculture was investigated. The minimum inhibitory concentration (MIC) of ampicillin, ceftiofur, gentamicin, neomycin, streptomycin, enrofloxacin, florfenicol, oxytetracycline and trimethoprim-sulphamethoxazole was determined using broth microdilution method. Susceptibility to imipenem and detection of extended-spectrum β-lactamases (ESBLs) in ampicillin resistant strains was performed by combination disc diffusion method. Results revealed that resistance among environmental strains was rare. Among *Aeromonas* strains from clinical sources, acquired resistance to tetracycline and fluoroquinolone was detected and it could be possible that acquired resistance is associated with the use of antimicrobials in therapy.

Keywords: Aeromonas *spp.;* Vibrio *spp.;* P. shigelloides*; Philippines; Thailand; Antimicrobial resistance*

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1. Introduction

The role of *Aeromonas* spp., 'non-cholera vibrios' (NCVs) and *Plesiomonas shigelloides* in causing different types of infection has caused increased concern. This includes species of growing public health importance that are potentially pathogenic for humans and animals. .

The genus *Aeromonas* comprises 14 species, although the taxonomy has yet to be resolved [1]. *Aeromonas hydrophila*, *A. caviae, A. veronii* bv *sobria, A. jandaei* and *A. schubertii* are considered as opportunistic human pathogens [1-4]. *Aeromonas trota* has been isolated from human clinical sources although its role as a causative agent of human disease is not fully elucidated [5]. *Aeromonas eucrenophila* shows pathogenic potential as demonstrated by fluid accumulation in the rabbit ileal loop test after several passages [6]. *Aeromonas salmonicida* is an important pathogen of salmonids and carp causing furunculosis and ulcer disease [7]. Gastroenteritis caused by *Aeromonas* spp. is self-limiting and usually does not require medical treatment, however, may lead to fatality in immunocompromised individuals [8].

'Non-cholera vibrios' (NCVs) of clinical importance (pathogenic for humans) include *V. cholerae* Non-O1, V. *vulnificus, V. parahaemolyticus, V. mimicus and to a lesser extent, V. fluvialis, V. furnissii, V.* (*Grimontia*) *hollisae, V.* (*Listonella*) *damselae, V. algynolyticus, V. metschnikovii, V. cincinnatiensis* and *V. harveyi* (*V. charchariae*) [9-13]. These strains are able to colonise the surface and internal organs of marine vertebrates and invertebrates. Food poisoning caused by *V. parahaemolyticus* is associated with the consumption of raw or undercooked seafood [9]. *Vibrio vulnificus* may cause meningoencephalitis, necrotising wound infections, primary septicaemia and gastroenteritis [10,14].

Plesiomonas shigelloides could be considered as a newcomer among the expanding group of identified water- and food-borne pathogens. This organism is also implicated in both intestinal and extra-intestinal infections in humans and in cases of diarrhoea in animals [15-17].

Susceptibility to antibiotics differs among the species in the three genera, but strains are generally susceptible to broad-spectrum cephalosporins, aminoglycosides, quinolones, chloramphenicol and tetracyclines [18-20]. However, resistance to these antibiotics has also been reported [21-23].

The occurrence and isolation of these bacteria from different sources has been reported in Asia, including the Philippines and Thailand [24-27]. However, information on antimicrobial susceptibility of these isolates, especially those recovered from aquaculture and aquatic environments, is scarce [28]. The aim of this study was to acquire data on the occurrence of antimicrobial resistance among 38 strains of *Aeromonas* spp., NCVs and *P. shigelloides* isolated from different sources in the Philippines and Thailand. In addition, the presence of extended-spectrum β-lactamases (ESBLs) from selected strains was determined.

2. Materials and Methods

2.1. Bacterial strains

Thirty-eight strains belonging to *Aeromonas* spp. (n=9), NCVs (n=27) and *Plesiomonas shigelloides* (n=2) were used in the study. The distribution of these strains was as follows: *A. hydrophila* (n=8); *A. veronii* bv *sobria* (n=1); *Vibrio* spp. (non-pathogenic for humans) (n=11); *V. parahaemolyticus* (n=14); *V. cholerae* Non-O1, non O139 (n=2); and *P. shigelloides* (serotypes O77:H22 and NA:H21) (n=2). The isolates were collected from environmental and clinical sources in the Philippines (n=27) and Thailand (n=11): the sources and distribution are summarised in Table 1.

Isolates were originally identified from the country of source following local identification schema. Isolates that were gram negative, oxidase positive and glucose fermenting were further characterised using API 20E (bio Merieux, Marcy-l'Etoile, France). Strains identified as *Aeromonas* spp. were further classified by Aerokey II scheme of Carnahan et al. [29] and by the phenotypic scheme of Abbott et al. [30]. *Aeromonas* and *Vibrio* spp. strains were further identified and verified at the Microbial Diseases Laboratory, California Department of Health Services, California, USA. *Vibrio parahaemolyticus* strains were confirmed by thermolabile direct haemolysin (*tlh*) gene probe at the Gulf Coast Seafood Laboratory, Food and Drug Administration, Alabama, USA. *Vibrio cholerae* strains were confirmed by PCR and serotyped at the Swedish Institute for Infectious Disease Control, Solna, Sweden. *Plesiomonas shigelloides* strains were confirmed and serotyped at the Institute of Preventive and Clinical Medicine, Bratislava, Slovak Republic, according to the schemes of Shimada and Sakazaki [31] and the Plesiomonas International Antigenic scheme of Aldova and Schubert [32].

2.2 Antimicrobial susceptibility testing

A broth microdilution method (VetMIC™, National Veterinary Institute (SVA), Uppsala, Sweden) was used for susceptibility testing. The standards of the National Committee for Clinical Laboratory Standards (NCCLS) M31-A were followed with some modifications [33]. The direct inoculum method, with Mueller-Hinton broth (MHB, Difco, MD, USA) as test medium, was used and the microdilution panels were incubated at 28°C for 18 h. For isolates that did not grow, 2% NaCl was added to MHB. *Escherichia coli* ATCC 25922 was included as a quality control strain and *A. hydrophila* ATCC 35654, *A. caviae* ATCC 15467 and *V. harveyi* CCUG 28584 were included as reference strains.

Minimum inhibitory concentration (MIC) was registered as the lowest concentration of an antimicrobial agent completely inhibiting bacterial growth. As no criteria were available for these bacteria, the Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM 2002, SVA, Uppsala) susceptibility criterion for enterobacteriaceae was used [34]. The MIC results were interpreted using the following resistance breakpoints (mg/L): ampicillin: >8; ceftiofur: >2;

gentamicin: >8; neomycin: >8; streptomycin: >32; enrofloxacin: >0.5; florfenicol: >16; oxytetracycline: >8; and trimethoprim-sulphamethoxazole (TMPS): >2/38.

2.3 Detection of extended-spectrum β-lactamases (ESBLs)

Strains resistant to ampicillin (n=22) were tested for the presence of ESBLs by combination disc diffusion method. Discs of cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg) and imipenem (10 µg) were used in the detection, along with discs in combination with clavulanic acid: cefpodoxime (10/1 µg), ceftazidime (30/10 µg), cefotaxime (30/10 µg) (Oxoid, Hampshire, England). The tests were conducted according to NCCLS standard M100-S9 with some modifications [35]. Mueller-Hinton agar (Oxoid, Hampshire, England) was used and the plates were incubated at 28°C for 18 h. An ESBLs-producing organism was confirmed if there was a ≥5 mm increase in zone diameter for the antimicrobial agent tested in combination with clavulanic acid compared with the zone when tested without clavulanic acid. .

3. Results

The results for MIC determination and antimicrobial susceptibility are summarised in Table 2. Susceptibility to ampicillin revealed that 8/9 (89%) *Aeromonas* strains exhibited resistance, including seven strains of *A. hydrophila* and one strain of *A. veronii* bv *sobria*, which are clinical strains. An environmental strain of *A. hydrophila* was susceptible. Among the NCVs, all *Vibrio* spp. (non-human, environmental) were susceptible, while 12 strains of *V. parahaemolyticus* and both strains of *V. cholerae* Non-O1 were susceptible. Five strains of *A. hydrophila* and one strain of *A. veronii* bv *sobria* were resistant to ceftiofur, whereas all strains of NCVs were susceptible.

In the case of aminoglycosides (gentamicin, neomycin, streptomycin), florfenicol and TMPS all *Aeromonas* spp. and NCVs were susceptible. Two strains of *A. hydrophila* and one strain of *A. veronii* bv *sobria* were resistant to enrofloxacin; whereas four strains of *A. hydrophila* and one strain of *A. veronii* bv *sobria* were resistant to oxytetracycline.

Both strains of *P. shigelloides* were resistant to ampicillin, neomycin and streptomycin; and one strain (no. 14) was resistant to enrofloxacin, oxytetracycline and TMPS.

None of the ampicillin-resistant strains was ESBLs producing and all strains of *Aeromonas*, NCVs and *P. shigelloides* were susceptible to imipenem.

4. Discussion

In most developing countries including the Philippines and Thailand, the frequency of isolation of *Aeromonas* spp., NCVs and *P. shigelloides* is increasing and occurrences of antibiotic resistance have been reported [36-38].

Cases of traveller's diarrhoea are reported among foreign tourists [16, 39-40]. A report from Japan showed that NCVs, *Aeromonas* spp. and *P. shigelloides* were abundant in faecal samples collected from overseas travellers [39]. These bacteria are isolated from aquatic environments (fresh and marine), aquaculture, animals and humans [41-43]. Thailand and the Philippines are major aquaculture and marine exporters [28] and regularly frequented by foreign visitors.

Documentation of the antimicrobial susceptibility of these strains recovered from various sources in these countries is limited [28]. The current study determined the antimicrobial susceptibility profile of *Aeromonas* spp., NCVs and *P. shigelloides* isolated from various sources in the Philippines and Thailand, and procured information on the occurrence of antibiotic resistance among the various strains examined.

Most *Aeromonas* spp. are inherently resistant to ampicillin, including *A. hydrophila, A. caviae, A. veronii* bv *veronii* and *A. eucrenophila* [30,44]. *Aeromonas media*, *A. veronii* bv *sobria*, *A. salmonicida* and *A. trota* have varying ampicillin susceptibility, with the latter reported to be inherently susceptible to ampicillin [30, 44-46]. Resistance to ampicillin may assist in the identification of these species [30]. The results of this study confirmed the ampicillin susceptibility trait of *Aeromonas*. In this study, susceptibility to ampicillin could have been influenced by the sources of the strains. For instance, clinical strains of *A. hydrophila* were resistant to ampicillin whereas the environmentally recovered strain was susceptible. Resistance to ceftiofur, a third generation cephalosporin, was only observed among clinical strains of *Aeromonas* spp, including four strains of *A. hydrophila* and one strain of *A. veronii* bv *sobria.* Increasing resistance to third generation cephalosporin has also been reported among strains of *Aeromonas* spp. isolated from clinical sources [47].

Based on the results of this study, it appeared that aminoglycosides (gentamicin, neomycin, streptomycin), florfenicol (chloramphenicol derivative) and TMPS were the most potent antimicrobials against these bacteria, as all strains of *Aeromonas* spp displayed *in vitro* susceptibility to these antimicrobials. *Aeromonas* spp. are usually susceptible to most aminoglycosides [48], however resistance to several aminoglycosides such as streptomycin, gentamicin, tobramycin and amikacin have also been reported [21,44,47]. An increased resistance to TMPS is observed among clinical strains of *Aeromonas* isolated from Taiwan [47], whereas most strains are susceptible to chloramphenicol and resistance is less common [48]. All strains were susceptible to florfenicol, a chloramphenicol derivative that is approved for veterinary use.

Although fluoroquinolones are used for treating *Aeromonas* infections in humans [48], in this study two strains of *A. hydrophila,* recovered from patients with diarrhoea and one strain of *A. veronii* bv *sobria* were resistant to enrofloxacin, a fluoroquinolone used for animals. Previous studies [44,47] have determined that *Aeromonas* strains are mostly susceptible to this antibiotic, however, the results from the current study indicated that these strains have developed resistance to this antimicrobial. In the case of oxytetracycline, four strains of *A. hydrophila* and one

strain of *A. veronii* bv *sobria* were resistant. Resistance to tetracycline among *Aeromonas* spp. varies [21,44], however an increasing resistance to this antibiotic has been reported in Taiwan [47] and in Thailand [38]. A summary report from Thailand indicates that most *A. hydrophila* and *A. veronii* bv *sobria* strains are resistant to oxytetracycline, which is commonly used as a prophylactic and for treatment in aquaculture. Due to this increase in resistance, modern antimicrobials, such as fluoroquinolones, have largely replaced tetracyclines in Asian aquaculture [38,49].

Resistance among NCVs was less prevalent compared with *A. hydrophila* clinical strains in this study. Among the NCVs, 12 strains of *V. parahaemolyticus* were resistant to ampicillin. This finding supported the widespread β-lactam resistance among vibrios from different sources [50-52]. In contrast, all strains of *Vibrio* spp. (n=11) from environmental sources and both strains of *V. cholerae* Non-O1 were susceptible to ampicillin, although, resistance to this antibiotic is reported among *V. cholerae* Non-O1 strains [53-54]. This study also determined that NCVs were susceptible to the majority of antimicrobials tested, indicated by the susceptibility of all the strains to ceftiofur, aminoglycosides, florfenicol, TMPS, enrofloxacin and oxytetracycline. However, an increasing number of cases of resistance to these antimicrobials have been reported [51,55].

Both *P. shigelloides* strains were resistant to the majority of antibiotics, which included ampicillin, neomycin and streptomycin and one strain (no.14) was resistant to enrofloxacin, oxytetracycline and TMPS. These results confirmed previous studies where *P. shigelloides* strains are resistant to ampicillin, carbenicillin, kanamycin and streptomycin and susceptible to gentamicin, nalidixic acid and tetracycline [23,56-58]. *Plesiomonas shigelloides* as previously reported are inherently resistant to ampicillin and are naturally susceptible or intermediate to tetracyclines, several aminoglycosides, quinolones, TMPS and chloramphenicol [57]. However, some strains may carry resistant genes to tetracycline [58].

Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum (third-generation) cephalosporins. The presence of ESBLs was reported in enterobacteriaceae and *Pseudomonas aeruginosa* [59-60]. In this study, production of ESBLs among *Aeromonas* spp., NCVs and *P. shigelloides* was not demonstrated.

Based on the results of this study, antimicrobial resistance among environmental strains was rare. Some strains although previously reported as susceptible now appear to be developing resistance. Acquired resistance to tetracycline and fluoroquinolone was demonstrated among *Aeromonas* strains from clinical sources, and it could be possible that this acquired resistance is associated with the use of antimicrobials in therapy.

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Table 1. *Distribution and sources of* Aeromonas *spp., NCVs and* P. shigelloides

No.	Species	Country	Source
1	*A. hydrophila*	Thailand	human patient
4	*A. hydrophila*	Thailand	human patient
5	*A. hydrophila*	Thailand	human patient
6	*A. hydrophila*	Thailand	human patient
7	*A. hydrophila*	Thailand	human patient
8	*A. hydrophila*	Thailand	human patient
13	*A. hydrophila*	Thailand	nile tilapia (*Oreochromis nilotica*) liver
17	*A. hydrophila*	Philippines	source/creek water
10	*A. veronii* bv. *sobria*	Thailand	spotted ghost catfish(*Ompok bimaculatus*) liver
34	*Vibrio* spp.	Philippines	pond sediment
47	*Vibrio* spp.	Philippines	reservoir water
49	*Vibrio* spp.	Philippines	reservoir water
58	*Vibrio* spp.	Philippines	pond water
61	*Vibrio* spp.	Philippines	reservoir water
68	*Vibrio* spp.	Philippines	reservoir water
69	*Vibrio* spp.	Philippines	source water
70	*Vibrio* spp.	Philippines	source water
71	*Vibrio* spp.	Philippines	source water
72	*Vibrio* spp.	Philippines	source water
75	*Vibrio* spp.	Philippines	rearing water of *Penaeus monodon*
16	*V. parahaemolyticus*	Philippines	source/creek water
18	*V. parahaemolyticus*	Philippines	pond water
19	*V. parahaemolyticus*	Philippines	reservoir water
41	*V. parahaemolyticus*	Philippines	Crab haemolymph
44	*V. parahaemolyticus*	Philippines	pond water
51	*V. parahaemolyticus*	Philippines	source/creek water
54	*V. parahaemolyticus*	Philippines	source/creek water
67	*V. parahaemolyticus*	Philippines	source/creek water
77	*V. parahaemolyticus*	Philippines	rearing water of *P.monodon*
90	*V. parahaemolyticus*	Philippines	*P. monodon*
92	*V. parahaemolyticus*	Philippines	*P. monodon*
96	*V. parahaemolyticus*	Philippines	Crab haemolymph
99	*V. parahaemolyticus*	Philippines	*P. monodon*
100	*V. parahaemolyticus*	Philippines	*P. monodon*
12	*V. cholerae* Non-O1	Thailand	hybrid catfish (*Clarias macrocephalus*) liver
15	*V. cholerae* Non-O1	Philippines	source/creek water
11	*P. shigelloides* O77:H22	Thailand	hybrid catfish (*Clarias macrocephalus*) kidney
14	*P. shigelloides* NA:H22	Thailand	striped catfish (*Pangasius sutchi*) liver

Table 2. *Distribution of MICs for the examined bacteria. Shaded areas indicate resistance*

MIC (mg/L)	*A. hydrophila*	*A. veronii* bv *sobria*	*Vibrio* spp.[1]	*V. parahaemolyticus*	*V. cholerae* Non-O1	*P. shigelloides*
Number	8	1	11	14	2	2
Ampicillin						
≤1	1	0	8	0	0	0
2	0	0	2	1	0	0
4	0	0	0	1	1	0
8	0	0	1	0	1	0
>8	7	1	0	12	0	2
Ceftiofur						
≤0.25	0	0	4	1	0	0
0.5	1	0	7	3	0	0
1	1	0	0	9	0	0
2	1	0	0	1	2	1
4	0	0	0	0	0	1
8	1	0	0	0	0	0
16	1	0	0	0	0	0
>16	3	1	0	0	0	0
Gentamicin						
≤2	7	1	11	8	2	0
4	1	0	0	6	0	0
8	0	0	0	0	0	2
16	0	0	0	0	0	0
>16	0	0	0	0	0	0
Neomycin						
≤4	7	1	11	14	2	0
8	1	0	0	0	0	0
16	0	0	0	0	0	2
32	0	0	0	0	0	0
>32	0	0	0	0	0	0
Streptomycin						
≤4	0	0	1	0	0	0
8	4	0	8	1	0	0
16	3	1	2	9	2	0
32	1	0	0	4	0	0
>32	0	0	0	0	0	2*

Table 2. *Distribution of MICs for bacterial strains examined. Shaded* (Continuation)

MIC (mg/L)	*A. hydrophila*	*A. veronii* bv *sobria*	*Vibrio* spp.[1]	*V. parahaemolytiucs*	*V. cholerae* Non-O1	*P. shigelloides*
Number	8	1	11	14	2	2
Enrofloxacin						
≤0.12	6	0	9	1	2	1
0.25	0	0	2	12	0	0
0.5	0	0	0	1	0	0
1	1	1	0	0	0	0
2	0	0	0	0	0	0
4	0	0	0	0	0	0
>4	1	0	0	0	0	1
Florfenicol						
≤2	7	1	11	14	2	2
4	1	0	0	0	0	0
8	0	0	0	0	0	0
16	0	0	0	0	0	0
>16	0	0	0	0	0	0
Oxytetracycline						
≤1	3	0	11	14	2	1
2	0	0	0	0	0	0
4	0	0	0	0	0	0
8	1**	0	0	0	0	0
16	0	0	0	0	0	0
32	2	1	0	0	0	0
64	2	0	0	0	0	1
>64	0	0	0	0	0	0
TMPS						
≤0.5/9.5	7	1	10	14	2	0
1/19	1	0	1	0	0	0
2/38	0	0	0	0	0	1
4/76	0	0	0	0	0	1
>4/76	0	0	0	0	0	0

*= ≥256; **=>8

[1] Species that are pathogenic for humans were ruled out using 25 biochemical tests that include the following species: *V. cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus, V. fluvialis, V. furnissii, V.* (*Grimontia*) *hollisae, V.* (*Listonella*) *damselae, V. alginolyticus, V. metschnikovii, V. cincinnatiensis, V. harveyi* (*V.charchariae*)

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APPENDIX

Potential virulence determinants of *Aeromonas* spp., *Vibrio* spp. and *Plesiomonas shigelloides* isolated in the Philippines and Thailand

This paper was presented as a poster during the Society for General Microbiology (SGM) 154th Meeting held in Bath, United Kingdom from 29 March to 02 April 2004.

Potential virulence determinants of *Aeromonas* spp., *Vibrio* spp. and *Plesiomonas shigelloides* isolated in the Philippines and Thailand

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Introduction:

- *Aeromas* spp., *Vibrio spp*., and *P. shigelloides* are increasingly regarded as significant pathogens of humans & animals
- We studied some of the potential virulence determinants of these organisms:
  - production of exototoxins (**cytotoxin & haemolysin)**
  - *in vitro* **adhesion** to human intestinal (Henle 407) cells
  - presence of haemolysin genes (***tlh, tdh, trh***)

- A total of 38 strains were collected from the Philppines (27) & Thailand (11)
- Sources of these strains include aquaculture/aquatic environments (32) & human diarrhoeal cases (6)
- The strains include:

A. hydrophila (8)	*Vibrio* spp. (11)
A. veronii bv *sobria* (1)	*V. parahaemolyticus* (14)
P. shigelloides (2)	*V. cholerae* Non 0-1 (2)

Methods:

▪Cytotoxicity assay

-Vero cell monolayers were exposed to several dilutions of bacterial culture filtrates

▪Haemolysis

Cell-associated: strains were streaked on BAP

Cell-free: bacterial culture filtrates were pipetted on punched wells on BAP

➢*Complete haemolysis was recognised as ß-haemolysis*

▪Thermostability assay

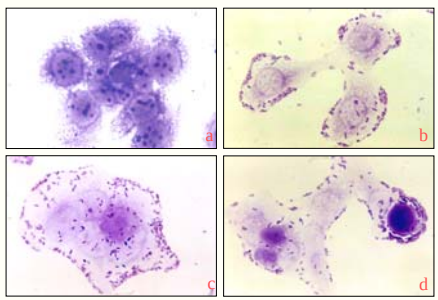
- bacterial culture filtrates were heated at 56°C for 15 min & at 80°C for 10 min

▪Adhesion

-Adhesion of *A. hydrophila* & *Vibrio* spp. to human intestinal (Henle 407) cells was determined

▪Haemolysis genes

-*tlh*, *tdh* & *trh* genes in *V. parahaemolyticus* were determined by DNA gene probes



a. Normal Henle 407 cells; b-d. *Aeromas hydropila* adhering to Henle 407 cells

a. Normal Vero cell; b. Moderate cell destruction; c. Total cell destruction & death

❑Strong association of virulence factors in clinical strains recovered from human diarrhoeal patients & diseased fishes, whereas lacking in environmental strains.

❑It is probable that these potential virulence determinants could be involved in the pathogenesis of the disease in humans & animals.

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Results:

- *A. hydrophila* (7/8), *A. veronii* bv *sobria* (1/1) & *V. cholerae* Non O-1 (2/2) were ß-haemolytic & cytotoxic on Vero cell monolayer.
- *Vibrio* spp. (11/11), *V. parahaemolyticus* (14/14), and *P. shigelloides* (2/2) were non-haemolytic & non-cytotoxic
- Strains from human diarrhoeal patients & diseased fishes were all ß-haemolytic & cytotoxic
- Haemolytic & cytotoxic activities were lost after exposure at 56°C for 15 min & at 80°C for 10 min
- All *A. hydrophila* (7/7) strains from human diarrhoeal patients adhered to Henle 407 cells with ≥30 bacterial cells/Henle cell
- All strains from aquaculture/ aquatic environments were not able to adhere to Henle 407 cells
- All *V. parahaemolyticus* (14/14) strains were *tlh*+; *tdh*, *trh* & urease –