CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

With an estimated 120,000 cholera associated deaths per year it must be considered one of the most important diarrheal diseases of our times, having a huge impact on social and economic aspects of regions (Halpern *et al*., 2004). Developing countries where socio-economic conditions are poor, sanitary and water supply systems not sufficient, and the level of personal hygiene low are the most severely affected by epidemic cholera. Cholera is mostly transmitted to humans by the ingestion of contaminated water and food, for prevention it is therefore of the utmost importance to have sustainable wastewater treatment, and safe drinking water systems in place. South Africa has not escaped the cholera onslaught, and has recently suffered a major epidemic. From January 2000 to December 2003 there were close to 130 000 cases of cholera reported in South Africa, with a total of 396 deaths (Department of Health, South Africa. 2004).

Cholera promoted sanitary reform world-wide not only because of the sudden onset of symptoms followed by death for the untreated patient, but also because of the striking success that was achieved in eliminating cholera epidemics through improvement of water supply and water sanitation. In developing countries cholera epidemics still pose a serious health risk, as the infrastructure for water sanitation is inadequate and the level of personal hygiene low. Therefore the causative agent of cholera, *Vibrio cholerae*, is to this

day still a popular research model, not only because of the ongoing epidemics but also because of the temporal and spatial diversity found within and between *V. cholerae* populations.

2.2 THE BACTERIUM

Vibrio cholerae is a facultatively anaerobic, asporogenous, motile, gram-negative rod. All members of the genus are oxidase positive. *Vibrio cholerae* requires, or at least prefers NaCl in their growth medium and for optimal growth it needs 5 to 15mM NaCl in the growth media. When grown in media not containing salt the bacteria will still grow, but with only 50% of the efficiency compared to growth in salt containing media. *V. cholerae* can survive in an environment with a pH ranging from 6 to 11, but they prefer to grow in alkaline conditions (Farmer and Hickman-Brenner 1992.).

Close to 200 serogroups (Yamai *et al.,* 1997) have been identified, but only the O1 and O139 serogroups pose a serious health threat. Cholera toxin is only known to be produced by strains belonging to these two serogroups. The production of CT (cholera toxin) is an essential determinant for virulence. Not all *V. cholerae* O1 strains produce cholera toxin, but even those that do not (as well as strains belonging to other serogroups) may still cause diarrhea, though not as serious as the CT producing strains (Levine *et al*., 1988.). This can be attributed to many other virulence factors, which these bacteria can employ (Kaper et al., 1994).

Isolates of *V. cholerae* O1 can be divided into two biotypes: El Tor and Classical, and three serotypes: Ogawa, Inaba and Hikojima (Table 1). The first epidemics were mostly attributed to Classical biotype strains, but since the seventh pandemic, the El Tor biotype has become predominant in epidemics (Blake, P.A., 1994). The differentiation of *Vibrio cholerae* O1 into biotypes is not required for the treatment of patients, but may be of epidemiological importance in helping to identify the source of infection. The three serotypes of *V. cholerae* are distinguished on basis of the designated antigenic formulas – AB, AC and ABC (Ogawa, Inaba and Hikojima), with the A antigen common to all serotypes. Antigenic shifts have been known to occur between Ogawa and Inaba, even within an epidemic (Kay *et al*., 1994), therefore serotyping has limited use as an epidemiological tool. Serotyping is still widely used because identification of the serotype with mono-specific antisera is regarded by many as the definitive serological confirmation test for *Vibrio cholerae* isolates. With O139 strains (having no described serotypes) being found to cause epidemics serotyping may become less important.

Table 1. Classification of epidemiological important *V. cholerae* strains.

Serogroups	***Biotypes***	***Serotypes***
O1	Classical and El Tor	Inaba Ogawa Hikojima
O139	El Tor	None Described

2.3 CHOLERA

2.3.1 THE DISEASE

Infection with Cholera Toxin producing *Vibrio Cholerae* (limited to serogroups O1 and O139) can cause watery diarrhea of such intensity that hypotensive shock and subsequent death can occur within 12 hours of the appearance of the first symptoms. Due to the ability of *Vibrio cholerae* to spread rapidly numerous cases are usually reported in the same community, which can lead to subsequent epidemics. Because of the large number of people infected, and the severity of the disease, cholera epidemics can have a huge impact on social and economic aspects of regions.

The incubation period of cholera can range from several hours to 5 days, and is dependent on inoculum size. The onset of the illness may be sudden, with profuse watery diarrhea, or there may be initial symptoms like abdominal discomfort and simple diarrhea. Mucus in the stool gives the ‘rice water’ appearance that is generally associated with cholera. Vomiting is often present, occurring a few hours after the onset of the diarrhea. In the most severe form, termed Cholera gravis, the diarrheal rate may reach 500 to 1000 ml/h, leading to tachycardia, hypotension, and vascular collapse due to dehydration. Severe dehydration can lead to death within hours of the onset of symptoms, unless fluids and electrolytes are rapidly replaced (Bennish, M.L., 1994).

2.3.2 CHOLERA: THE SITUATION IN SOUTH AFRICA

The first case of cholera in South Africa was diagnosed in 1973, with another seven confirmed cases in the period up to October 1980. The first case diagnosed in an open community was on the 2nd October 1980 in a hospital in the vicinity of Malelane, and was followed by the first South African epidemic. From October 1980 to July 1987 25,251 cases were confirmed in the Malelane region, which is situated close the Mozambique border. In the same period (1983) there was an undocumented outbreak of cholera in the Kwazulu-Natal province. Cholera seemed to be under control since 1987 with few cases being reported until 2000, when the most severe epidemic struck. In the period starting August 2000 and ending in December 2003 128,514 confirmed cases, with 396 deaths, were reported in South Africa, with Kwazulu-Natal and the Eastern Cape being the foci of the epidemic. Cholera cases have been declining since 2001, as can be seen in the following table (Table 2), the current status seems to indicate that the epidemic has subsided (KZN Health 2004, Department of Health, South Africa 2004).

Table 2. Confirmed Cholera Cases 2000-2003

Period	***Confirmed Cases***	***Total Deaths***
15.08.2000 – 31.07.2001	106 389	229
01.08.2001 – 31.12.2002	18 224	122
01.01.2003 – 31.12.2003	3901	45
TOTAL	128 514	396

2.4 VIRULENCE FACTORS

2.4.1 CHOLERA TOXIN

The genes encoding Cholera Toxin (*ctxAB)* are part of a larger genetic element (*ctx* genetic element) consisting of at least six genes: *ctxAB, zot, ace, cep* and *orfU*. These genes make up the core region that is flanked by two or more copies of a repeat sequence (Trucksis *et al*., 1993; Pearson *et al.,* 1993.). It has been demonstrated that in *Vibrio cholerae* O1 (Strain P27459) the entire *ctx* element constitutes the genome of a filamentous bacteriophage, CTXϕ (Waldor and Mekalanos, 1996). Not all *V. cholerae* strains have the genetic ability to produce cholera toxin, with only some O1, O139 and O141 serotype strains found to harbor the required genes (Yamai *et al*., 1997). It is speculated that non-cholera toxin (CT) producing strains can be converted to CT producing strains by the addition of the *ctx* genetic element during CTXϕ invasion. The receptor required by CTXϕ for the invasion of *V. cholerae* is the toxin co-regulated pili (TCP) (Waldor and Mekalanos, 1996). Faruque *et al.* (1998) showed that 10 *ctx*-negative *Vibrio cholerae* O1 strains carrying the TCP pathogenicity island (coding for TCP) could be infected by CTXϕ in vitro, or in the intestines of mice. Furthermore they found that 135 out of 136 TCP-negative O1 strains could not be invaded by the phage, indicating the importance of TCP as the receptor.

Finkelstein and LoSpalluto (1969) were the first researchers to purify the cholera toxin from *Vibrio cholerae* in 1969. This allowed the investigation of the fundamental properties of the cholera toxin, such as structure and function. The cholera toxin is a sub-

unit toxin consisting of an A and B sub-unit. The A-sub-unit possesses a specific enzymatic activity, while the B-sub-unit binds the holotoxin to the eukaryotic cell receptor. Neither the A nor the B sub-units can cause disease symptoms individually. The B-sub-unit contains 103 amino acids and has a weight of 11.6 kDa. The mature A-sub-unit has a mass of 27.2 kDa and is cleaved proteolytically to yield two polypeptide chains: A1 (195 residues, 21.8 kDa), and A2 (45 residues, 5.4 kDa). The ratio of B-sub-units to A-sub-units in cholera toxin is 5:1.

The actions of the toxin are well studied, and although not all mechanisms are fully understood there is considerable insight in its pathogenesis. The B-sub-unit binds to the ganglioside GM1 receptor. Binding of the toxin requires that at least two of the five B-sub-units interact with the GM1. After binding, the A-sub-unit is translocated across the membrane, how this is achieved is still unknown. Translocation requires the reduction of the disulfide bond between the A1 and A2 sub-units. It is speculated that the A2 sub-unit does not enter the cell. Once inside the cell the cholera toxin (CT) catalyzes the transfer of the ADP-ribose moiety of NAD to the alpha sub-unit of the Gs protein. The Gs protein then activates adenylate cyclase, which in turn mediates the transformation of ATP to cyclic AMP (cAMP). The cAMP is an intracellular messenger for a variety of cellular pathways, and activates a cAMP-dependant protein kinase A, leading to protein phosphorylation, altering of ion-transport (increased Cl ion secretion), and ultimately to diarrhea. The exact mechanism is not completely understood and it is speculated that this mechanism involves prostaglandins and interactions with the enteric nervous system as well as the immune system (Kaper *et al*., 1994; Sprangler *et al.*, 1992).

2.4.2 OTHER TOXINS PRODUCED BY *V. CHOLERAE*

Vibrio cholerae strains that do not produce cholera toxin can still cause diarrhea in volunteers and thus researchers found that the bacterium has an arsenal of alternative toxins that it can employ. Genes encoding Hemolysin-Cytolysin (*hly*A) are present in Classical, El Tor and Non-O1 strains. The *hlyA* genes present in El Tor and Non-O1 strains are essentially identical, while Classical strains carry a distinct *hlyA* gene (Honda *et al*., 1979). The active cytolysin has a mass of 65 kDa, and has been shown to cause fluid accumulation and bloody diarrhea in rabbits (Ichinose *et al.*, 1987). In 1991 Fassano *et al.* reported a novel *Vibrio cholerae* toxin, ZO Toxin, that increases permeability of the small intestinal mucosa by affecting the structure of the zonula occludens. The ZO toxin is speculated to be responsible for diarrhea and as well as other symptoms associated with non-CT producing *V. cholerae* strains (Fassano *et al*., 1991). Another recently identified *V. cholerae* enterotoxin, Accessory cholera toxin (Ace), was identified by Trucksis *et al.* (1993). Ace was found to significantly increase Cholera Toxin associated fluid accumulation in ligated rabbit ileal loops, indicating that Ace is an accessory virulence factor in the pathogenesis of CT producing strains. The occurrence of various other toxins have been reported in non-CT producing *Vibrio cholerae* strains, amongst them Shiga-like toxin, Heat-Stable Toxin, New Cholera Toxin, Sodium Channel Inhibitor and Thermostable Direct Hemolysin (Ogawa *et al*., 1990; Sanyal *et al*., 1983; Tamplin *et al*., 1987; Honda *et al*., 1986). The precise role of toxins other than that of CT in cholera is not known. The additional toxins cannot cause severe epidemic diarrhea, but may contribute to some of the symptoms experienced by a cholera-affected person.

2.5 *VIBRIO CHOLERAE* IN THE ENVIRONMENT

Until the late 1970's *V. cholerae* was believed to be a highly host adapted bacterium and incapable of surviving longer than a few hours outside the host. More recent studies have however shown that *V. cholerae* can survive for long periods in laboratory microcosms-water (Islam, 1990). Data accumulated over the past decade shows that *V. cholerae* is an autochthonous inhabitant of brackish water and estuarine systems (Xu *et al.*, 1982; Colwell and Huq, 1994). Colwell *et al.* found that *V. cholerae* can be more readily isolated from aquatic systems when the water temperature is higher than 17 °C and the salinity between 0.2 and 2.00% (Colwell and Huq, 1994). Jiang et al. (2000 a) also noted the importance of these factors in *V. cholerae* isolations. Several other factors influencing survival including the level of water pollution, water pH, the association with zooplankton and Chironomid egg masses as reservoirs have been reported (Huq *et al.*, 1983; Huq *et al.*, 1984; Halpern *et al.*, 2004). Huq *et al.* (1995) demonstrated that *V. cholerae* remained culturable for longer periods in Laboratory microcosm water when associated with live planktonic copepods, compared to dead copepods. Halpern *et al.* (2004) showed a correlation between water temperature, Chironomid egg masses and *V. cholerae* isolations in fresh water habitats. It was proposed that *V. cholerae* might utilise the nutrient rich egg masses as growth substrate. The factors influencing active propagation of *V. cholerae* in the environment is still a popular research topic, and new knowledge is gained on a continuous basis.

Vibrio cholerae can survive in nutrient deprived environments in a dormant or viable but non-culturable (VNBC) state in environments of nutrient deprivation. The bacteria thus do not necessarily die within the aquatic environment, but survive in a manner that allows it to be infectious under favourable conditions. These aquatic *V. cholerae* strains can not be isolated and cultured, but when inoculated into rabbit ileal loops they do cause fluid accumulation. The bacteria survive better in aquatic systems where the temperature is above 10°C. The bacteria undergo physical changes associated with conversion to the VNBC state, such as becoming ovoid and reduced in size. These cells do not grow on standard laboratorium media but have been shown to be metabolically active. Experiments showed that under nutrient deprivation conditions *V. cholerae* cells will become spherical and smaller within 20 days, these cells can then survive in a semi-dormant state for long periods. With addition of nutrients these cells can regain their culturable state within two hours. The change to the VNBC state is also accompanied by a decrease in lipid, carbohydrate, protein and DNA content at a macromolecular level (Colwell and Huq, 1994; Huq *et al.*, 1990; Roszak and Colwell, 1987).

2.6 IDENTIFICATION OF *VIBRIO CHOLERAE*

2.6.1 ENRICHMENT

Investigators agree that enrichment enhances the isolation of *Vibrio cholerae* from convalescent patients and the environment. Alkaline peptone water (APW) is the most widely used enrichment broth for *Vibrio cholerae* isolation. Compared to other bacteria *Vibrio* sp. grows rapidly in APW media, hence they will be present in greater numbers

than non-*Vibrio* organisms within 6 to 8 hours. Other enrichment broths such as Monsur's Trypticase tellurite taurocholate-peptone (TTP) have also been formulated. TTP has been reported to increase the isolation of *V. cholerae* compared to APW owing to the inhibition of non-*Vibrios*. The use of selective media such as TTP may however not offer the advantage of a short incubation time, as with APW (Farmer and Hickman-Brenner, 1992; Baumann *et al*., 1984).

2.6.2 SELECTIVE PLATING MEDIA

Vibrio cholerae will grow on a variety of commonly used agar media, but isolation from certain samples is more easily accomplished by the use of selective plating media. Several specialized selective media have been developed for *Vibrio cholerae*, as the most routine enteric screening media are unsuitable for the isolation of these organisms. These specialized media include thiosulfate citrite bile salts sucrose Agar (TCBS), Tellurite taurocholate gelatin agar (TTGA), and Vibrio agar. TCBS and TTGA are the two most commonly used and the most widely studied selective plating media for *V. cholerae*. In various studies the efficacies of TCBS and TTGA for the isolation of *V. cholerae* O1 were reported as essentially equal (Kay *et al*., 1994).

TCBS is probably the most widely used selective plating media for Vibrios. Overnight growth on TCBS will produce large slightly flattened yellow colonies (non-sucrose-fermenting organisms such as *V. parahaemolyticus* will produce green colonies). It is available commercially, does not require autoclaving and is highly selective. TCBS is, however, subject to brand-to-brand variation in selectivity and growth. TCBS is also not

suitable for combination with some direct diagnostic tests. This medium is also expensive, especially when working with many isolates (Farmer and Hickman-Brenner, 1992; Baumann *et al*., 1984).

TTGA (also known as Monsur's agar) is inexpensive to prepare and allows tests like oxidase reaction and slide agglutination to be done directly from the plate. Overnight growth of *Vibrio cholerae* on TTGA appears as small opaque colonies with slightly darkened centers. Colonies eventually turn gunmetal grey. TTGA is not commercially available and requires autoclaving during its preparation. Since many members of the genus *Vibrio* have similar characteristics on TTGA, isolates suspected of being *Vibrio cholerae* must be examined by using biochemical tests or antisera (Kay *et al*., 1994).

2.6.3 BIOCHEMICAL IDENTIFICATION OF *V. CHOLERAE*

Biochemical identification of *Vibrio cholerae* may be accomplished by using a number of tests as is listed in Table 3. Whenever test results correlate with those in the table, the isolate can be confidently identified as *Vibrio cholerae.* It is therefore advisable to screen isolates resembling *V. cholerae* on selective plating media before performing comprehensive biochemical tests. This aids in reducing the number of non-*Vibrio cholerae* isolates, saving time and expenses. Kligler's Iron Agar (KIA), Triple Sugar Iron Agar (TSI), arginine, lysine, oxidase, or string tests can be used in the initial screening phase. KIA and TSI are carbohydrate-containing media widely used for screening enteric pathogens, these media can differentiate bacteria on their ability to ferment glucose, lactose and/or sucrose, and to reduce sulfur to hydrogen sulfide. With KIA the reaction

should be Alkaline/Acidic (lactose negative/glucose fermentation), no gas, no hydrogen sulfide, and with TSI Acidic/Acidic (Glucose and lactose and/or sucrose fermentation), no gas, no hydrogen sulfide. With the string and oxidase tests *V. cholerae* can be differentiated from closely related *Aeromonas* and *Enterobacteriaceae* spp., *V. cholerae* is string test positive, while *Aeromonas* and *Enterobacteriaceae* are usually negative. *Vibrio, Neisseria, Campylobacter, Aeromonas, Plesiomonas, Pseudomonas,* and *Alcaligenes* are all oxidase positive, while all the *Enterobacteriaceae* are oxidase negative. Arginine, Lysine, and Ornithine broths may be suplemented with 1% NaCl to enhance growth of *V. cholerae*. The reaction should be positive within two days for Lysine and Ornithine decarboxylase, but if negative should be incubated for up to seven days, *V. cholerae* is typically negative for Arginine dihydrolase (Kay *et al.,* 1994; Baumann *et al*., 1984).

Table 3. Biochemical identification of *Vibrio* spp.

Test	***V. cholerae***	***V. mimicus***	Halophilic ***Vibrios***	***Aeromonas hydrophila***	***A. veronii***	***Plesiomonas shigelloides***	***Entero-bacteria***
KIA	K/A	K/A	V	V	K/AG	K/A	V
TSI	A/A	K/A	V	V	A/AG	K/A	V
String	+	+	+	-	-	-	-
Oxidase	+	+	+	+	+	+	-
Gas from glucose	-	-	-	+	+	-	V
Sucrose	+	-	V	V	+	-	V
Lysine	+	+	V	V	+	+	V
Arginine	-	-	V	+	-	+	V
Ornithine	+	+	V	-	+	+	V
VP	V	-	V	V	+	-	V
Growth in 0% NaCl	+	+	-	+	+	+	+
Growth (1% NaCl)	+	+	+	+	+	+	+

Where:
+ --------Positive
- --------Negative
V -------Variable reaction
K -------Alkaline (red color change)
A -------Acid (yellow color change)
G -------Gas produced (forms bubbles or cracks in KIA and TSI agar)

2.6.4 COMMERCIAL AND AUTOMATED SYSTEMS

Commercial systems have been developed that identify isolates on the basis of traditional biochemical tests. These tests are less laborious and give rapid results. Many of these systems (like API 20E) are still manual processes where only the data analysis is computerized, whilst other (like the VITEK) is a partially automated process.

2.6.4.1 API 20E

The API 20E (*bioMerieux*, Inc., Hazelwood, MO) system has become popular for rapid identification of members of the *Enterobacteriaceae* and other Gram-negative bacteria. The plastic strips consist of 20 small wells containing dehydrated media components. The bacterium to be tested is suspended in sterile saline solution and added to each well, the strip is then incubated for 16-24 hours and the colour reactions are noted as either positive or negative. The test results can be entered into a computer program to identify the bacterium. Initial evaluation of this system gave the indication that the accuracy of API 20E is equivalent to that of traditional biochemical methods ((Rutherford *et al.*, 1977), but later studies showed it to be less reliable (Holmes *et al.*, 1978; O'Hara *et al.*, 1992). These authors suggested that some specific test be replaced, and the incubation time for some tests be lengthened, in order to increase the accuracy.

2.6.4.2 VITEK

The VITEK is a fully automated bacteriology system that performs bacterial identification and antibiotic susceptibility testing analysis (bioMerieux, Inc., Hazelwood, MO). Compared to conventional methods that take up to 2 days to perform, this system can provide results within hours, making same-day reporting possible (Ling *et al.*, 2003). Spanu *et al.* (2003) found VITEK to be rapid and relatively accurate for the identification of Staphylococci, only faulting the identification of certain coagulase-negative species within this genus. One drawback of this system, is that this system could have difficulty in correctly distinguishing the closely related *Aeromonas* and *Vibrio* genera. A study has

shown that two *Aeromonas veronni* biovar *sobria* isolates were misidentified as *Vibrio alginolyticus* using the VITEK approach (Park *et al.*, 2003).

2.6.4.3 BIOLOG

The Biolog identification system (Biolog Inc., California, USA.) establishes identification based on the exchange of electrons produced during an organism's respiration. The exchange of electrons is visualized as a subsequent tetrazolium-based color change. The system tests the abililty of a microorganism to oxidize a panel of 95 different carbon sources. A database containing 434 species or groups of mostly gram-negative bacteria has been compiled by the manufacturer for Biolog associated identification. In a preliminary evaluation of the Biolog system, two out of three tested *V. cholerae* strains were correctly identified (Miller and Rhoden, 1991). In a later evaluation by Holmes *et al.* (1994) eight out of a possible ten *V. cholerae* isolates were correctly identified when automated scoring was done, with nine out of ten identified when scoring was done manually.

2.6.5 SEROLOGICAL IDENTIFICATION AND BIOTYPE DETERMINATION

Based on the thermostable polysaccharides that are part of the cell wall lipopolysaccharide there are currently just under 200 different specific O-antigens of *Vibrio cholerae* (Yamai *et al.*, 1997). *V. cholerae* strains belonging to different O-serogroups can not be biochemically differentiated, antiserum must be used to differentiate between the clinically important O1, O139 serotypes and the clinically less important 'non-O1'strains. Agglutination with polyvalent *V. cholerae* O1 antiserum is

sufficient proof to provide presumptive identification of *Vibrio cholerae* O1 (Kay *et al*., 1994). For the identification of O139 serogroup strains specific O139 antisera must be used.

The differentiation of *Vibrio cholerae* O1 into classical and El Tor biotypes is not necessary for the treatment of patients, but does play a role in helping identify the source of the epidemic. Biotyping is not appropriate for *V. cholerae* non-O1 strains, as tests can give atypical results if used with such isolates. At least two or more of the tests listed in Table 4 should be used to determine the biotype. The El Tor biotype was historically identified by its ability to hemolyse sheep erythrocytes, while classical strains were non-hemolytic, but by 1972 nearly all the El Tor isolates worldwide had become non-hemolytic. Exceptions are found among isolates from the U.S. Gulf coast and from Australia (Barret and Blake, 1981; Kay *et al*., 1994)

Table 4. Differentiation of Classical and El Tor biotypes

Biotype	VP test	Zone around polymixin B	Agglutination of Erythrocytes	Lysed by Classical IV phage	Lysed by El Tor Phage
Classical	-	+	-	+	-
El Tor	+	-	+	-	+

2.7 RAPID DIAGNOSTIC METHODS

Rapid diagnosis of *V. cholerae* is of importance to effectively control the spread of the disease. Several rapid detection techniques have been developed and are used for the identification of *Vibrio cholerae*.

2.7.1 DARK-FIELD AND PHASE-CONTRAST METHODS

In dark-field and phase-contrast microscopy liquid stools are examined for the presence of organisms with typical darting motility. The stools are examined with and without the addition of specific *V. cholerae* O1 antisera. If the addition of antisera results in the loss of mobility the test is regarded as positive. Diluents for the antisera and the stool must be carefully selected to avoid non-specific inhibition of mobility. The disadvantage of this method is the requirement for a dark-field or a phase-contrast microscope as well as a trained technician (Gustafsson and Holme, 1985).

2.7.2 IMMUNOFLUORESCENCE

Martinez-Govea *et al.* (2001) described the purification of bacterial outer membrane proteins (OMP), as well as the production of specific antisera and their use for fecal *Vibrio cholerae* antigen detection. The developed anti-OMP antisera showed high reactivity and specificity using enzyme-linked immunosorbent assay (ELISA), enabling the researchers to develop a rapid and inexpensive immunodiagnostic tool for the identification of *Vibrio cholerae*. Immunofluorescence techniques using antisera

conjugated to fluorescein isothiocyanate have also been reported to be able to detect *V. cholerae* O1 cells in a variety of specimen types (Kay *et al.,* 1994*).*

2.7.3 AGGLUTINATION

A commercially manufactured slide agglutination kit (Denka Seiken) has been developed for the serotyping of O1 isolates. This kit uses latex particles coated with monoclonal antibodies directed against the A, B and C antigens of *Vibrio cholerae* O1, and can also be applied to for the identification of *Vibrio cholerae*. During an investigation of an epidemic of cholera the kit was evaluated for its ability to confirm the diagnosis of cholera. Sixty-three percent of culture positive patients were correctly diagnosed by this kit as positive, while twelve percent of the culture negative patients yielded false positive tests with this system (Shaffer *et al*., 1989).

In coagglutination methods, antibodies against *Vibrio cholerae* are bound to the surface of *Staphylococcus aureus,* while retaining their binding capacity and specificity. This method has been reported by a number of authors to be able to rapidly diagnose cholera directly from stool or enrichment broths (Jesudason *et al*., 1984; Rahman *et al.,* 1987). Reports of the evaluation of this method, in a commercially prepared form, with culture collections in the United States have been encouraging (Colwell *et al*., 1992).

2.7.4 POLYMERASE CHAIN REACTION

In the polymerase chain reaction multiple copies of a specific portion of the bacterial genome is synthesized. The product can then be visualized and sized to determine if the targeted genome segment is present. Targeting specific genes common to all *Vibrio*

cholerae strains, rapid detection of all strains can be achieved. Toxigenic *Vibrio cholerae* O1 and O139 strains can be detected if the PCR is aimed at genes encoding for Cholera Toxin or associated virulence genes.

2.7.4.1 Detection of *Vibrio cholerae* - all strains

Nandi *et al.* (2000) designed a rapid method for species specific identification of *Vibrio cholerae* using primers targeted at the coding region of the Outer Membrane Protein (*ompW*). The distribution of genes for the Outer Membrane Protein and a Regulatory Protein (*ToxR*) were studied in *V. cholerae* as well as closely related organisms using primers and probes. PCR amplification showed that all strains of *Vibrio cholerae* tested positive for *ompW* and 98% of strains tested positive for *ToxR*. None of the non-*V. cholerae* strains belonging to the other *Vibrio* species produced amplicons with either of these genes. Restriction fragment length polymorphism (RFLP) analysis and nucleotide sequence data revealed that the *ompW* sequence is highly conserved among the *V. cholerae* strains belonging to different biotypes and serotypes. These results suggest that the *ompW* gene can be targeted for species specific identification of *Vibrio cholerae*. Doing a multiplex PCR with simultaneous amplification of the *ompW* and *ctxA* (coding for cholera toxin) provided additional information on the toxicity of the specific strains (Nandi *et al.*, 2000). Lyon (2001) reported a Taqman PCR for the detection of all *Vibrio cholerae* strains. The PCR used primers directed at a 70 bp target region of the non-classical *hlyA* gene, which has been shown to be present in, and specific for all *Vibrio cholerae* strains (Singh *at. al.*, 2001). This approach was tested on 60 different bacterial strains (compromising 21 genera) and this PCR assay was found to be positive for all

Vibrio cholerae strains and negative for all other screened bacterial strains. The developed PCR assay was found not only to be specific and sensitive, but also rapid due to the fact that no subsequent verification of amplicons needs to be carried out with the Taqman approach (Lyon, 2001).

2.7.4.2 Detection of enterotoxigenic *Vibrio cholerae*

Various approaches have been reported, with most studies targeting gene sequences within the *ctxAB* operon. This operon codes for cholera toxin, which is the major virulence factor of enterotoxigenic *Vibrio cholerae* strains. A number of researchers reported the detection of *V. cholerae* in stool samples and foods, with some suggesting it as an alternative diagnostic technique to DNA colony hybridization or enzyme linked immunosorbent assays (Fields *et al*., 1992; Kobayashi *et al*., 1990; Theron *et al*., 2000; Shirai *et al*., 1991; Koch *et al*., 1993; Karanasugar *et al*., 1995).

Several researchers reported multiplex PCR methods targeting the *ctx* operon as well as one or more additional virulence genes. Kapley *et al.* (2001) used primers targeting the *ctxA* and *tcpA* gene loci in a multiplex PCR, the developed assay was deemed to be a fast and sensitive screening technique to detect enterotoxigenic *V. cholerae*. Hoshino *et al.* (1998) reported the development of a multiplex PCR method for the rapid detection of toxigenic *V. cholerae* O1 and O139. This PCR amplifying a *ctx* and a *rfb* sequence, which were chosen to be specific for O1 and O139 strains. An amplicon generated from the rfb target sequence indicates the presence of O1 or O139 strains, while a ctx amplicon indicates the presence of cholera enterotoxin genes. The developed technique was able to

give insight into the epidemic potential of detected O1 and O139 strains. Using the multiplex approach Shangkuan *et al*. (1995) developed a PCR assay that could detect *Vibrio cholerae* O1 and assign it to biotypes. Enterotoxin-producing *V. cholerae* strains were identified with a primer pair that amplifies a fragment of the *ctxA2-B* gene, and strains were also simultaneously differentiated into biotypes with three primers specified for the *hlyA* gene in the same reaction. With this assay they could detect as low as three colony forming units (CFU's) per gram of food. Detection of *Vibrio cholerae* was not always the aim of PCR techniques targeting virulence genes, as various researchers used PCR to screen known *V. cholerae* strains for the occurrence of the different major and minor virulence factors (Singh *et al.,* 2002; Chow *et al*., 2001)

2.7.4.3 Primers for rapid identification of *V. cholerae* O139 serotypes.

All the genes of the *rfb* complex which encode the O antigen in the *V. cholerae* O1 El Tor strains have been found to be deleted in *V. cholerae* O139 (Stroeher *et al.,* 1995). In their place there is a new chromosomal region, which encodes for the lipopolysaccharide and capsular polysaccharide in *V. cholerae* O139 (Comstock *et al*., 1996). The capsular polysaccharide contains a unique sugar, a 3,6-dideoxy-hexose called colitose (similar to tyvelose). A primer-pair complementary to the gene encoding tyvelose was first used to generate a product of 720bp in *V. cholerae* O139. Based on the sequence of the obtained amplicon a new primer-pair for the detection of O139 serotypes was designed. These primers were then used in a study where pure bacterial cultures were submitted to PCR amplification. These primers generated an amplicon of 417bp from only *Vibrio cholerae* O139 Bengal strains (Falklind *et al*., 1996). In a later study the same PCR primer set was

used to screen 180 diarrheal stool specimens. All of the 67 *V. cholerae* O139 culture-positive stool specimens tested positive with the PCR, and the remaining specimens which contained either other recognized enteric pathogens or no pathogens, were all PCR negative. This PCR was also found to be equally effective testing either fresh or frozen samples (Albert *et al*., 1997).

2.8 TYPING METHODS

Control strategies for cholera depends on determining the origin and route of transmission of the disease. A need therefore exists for effective sub-typing methods that will allow the origin of strains to be traced. To understand the epidemiology and pathogenesis of *V. cholerae*, and their conversion from non-enterotoxin production to potential enterotoxin production, many studies still need to be done on how different strains of *V. cholerae* change over time and how genotypes differ according to geographical location. Typing studies may provide researchers with insight into some of the above-mentioned questions.

2.8.1 BACTERIOPHAGE TYPING

Bacteriophages have been used since the 1950's to differentiate among isolates of *V. cholerae*. Current phage systems are based on host-range as demonstrated by lysis of the bacteria. The first phage-typing system developed for *V. cholerae* divided classical isolates into five phage types using four different phages. With the advent of the seventh pandemic and the almost total replacement of Classical strains by El Tor strains several

other El Tor specific phage-typing systems had to be developed (Kay *et al.*, 1994). Nearly all phage-typing schemes now include phages specific for typing both Classical and El Tor *V. cholerae's*. The disadvantage of phage typing is that all current systems have limited discrimination. However a new phage-typing system has been proposed, this scheme is a modification of Basu's and Makerjee's El Tor scheme, with five new phages added to the five phages originally used (Chattopadaya *et al*., 1993). Phage typing methods can test large numbers of strains rapidly and the method is generally less laborious than molecular typing methods, but the propagation and maintenance of phages can be very demanding.

2.8.2 GENOTYPIC TYPING METHODS

The development of genotypic typing techniques has revolutionized the means by which bacterial isolates may be characterized. These methods are rapid to perform and flexible in their resolution and discrimination abilities. Several methods are available for the sub-typing of *Vibrio cholerae* strains and the most important of these will be discussed

2.8.2.1 Pulsed Field Gel Electrophoreses

Pulsed field gel electrophoreses (PFGE) separates large DNA fragments created by digestion of total genomic DNA with restriction endonucleases that cut DNA infrequently. The patterns generated by PFGE have been used in the analysis of a variety of bacterial organisms. Various studies have shown that PFGE is a typing technique with high resolution, which can be used with success on *Vibrio cholerae*. Choudhury *et al*. (1994) found that several different strains belonging to different serovars and biotypes

have distinct restriction patterns**.** Cameron *et al*. (1994) used PFGE to characterize 180 isolates of *Vibrio cholerae* O1. Isolates were digested by *NotI* and were separated into 63 patterns on the basis of band arrangements. This method was compared to multilocus enzyme electrophoreses (MLEE) and Ribotyping and it was found that it separated unrelated isolates more effectively. PFGE was shown to have better discrimination than a technique based on sequencing of a specific *Vibrio cholerae* toxin gene. *Vibrio cholerae* isolates that were presumed to be identical, on the base of DNA sequence of the cholera toxin B-subunit and multilocus enzyme electrophoreses markers, gave four different PFGE patterns in a study by Popovic *et al.* (1993.). The genetic diversity of *Vibrio cholerae* O1 strains from Argentina were determined by PFGE and random amplified polymorphic DNA (RAPD). It was found that these two methods gave comparable results, PFGE being the more reproducible technique in this study (Pichel *et al*., 2003.). Genotypic evolution in *Vibrio cholerae* O1 Bengal was suggested based on changes in PFGE patterns among isolates obtained from Bangladesh between 1993 and 1996 (Albert *et al.*, 1997).

PFGE is thought to be more rapid and less labour intensive than most other sub-typing techniques. The disadvantage of this technique is that in most cases a large number of bands are generated that poses problems when comparing results between different laboratories. Furthermore the variation detected by PFGE is often a result of genome instability (genome rearrangements etc. (Thal *et al*., 1997)). PFGE also cannot type all strains due to the occurrence of strains that exhibit endonuclease activity (Pichel *et al*., 2003).

2.8.2.2 Restriction Fragment Length Polymorphism based DNA fingerprinting

2.8.2.2.1 RFLP

In Restriction Fragment Length Polymorphism DNA fingerprinting (RFLP) genomic restriction fragments are produced using restriction enzymes, fragments are separated on agarose gels and detected using gene probes**.** Using gene probes to study RFLP patterns in the cholera toxin genes and their flanking sequences it was observed that clinical *Vibrio cholerae* isolates from the U.S. Gulf Coast region are different from other seventh pandemic isolates (Kaper *et al.,* 1982.) RFLP using rRNA genes probes (Ribotyping) has been used successfully for characterization and epidemiological studies of *V. cholerae* (Dalsgaard *et al.,* 1995; Popovic *et al*., 1993), this method is based on highly conserved rDNA sequences present as multiple copies in the genome of all bacteria, providing a good target for strain differentiation. Chromosomal DNA is digested with restriction enzymes, fragments are separated by gel electrophoreses and detected by cDNA probes directed at bacterial 16S and 23S rRNA genes. With ribotyping it was shown that clinical *V. cholerae* isolates from the Latin American epidemic that occurred in 1991 were related to seventh pandemic isolates from other parts of the world, suggesting that the Latin American epidemic was an extension of the seventh pandemic (Wachsmuth *et al*., 1991; Wachsmuth *et al*., 1993.). Ribotyping has been used to differentiate between phenotypically indistinguishable *V. cholerae* O1 strains, however the technique is labour intensive and experienced laboratory staff are required. Dalsgaard *et al*. (1999) tested the efficacy of an automated *Riboprinter* system against that of traditional ribotyping. Automated ribotyping using the *EcoRI* restriction endonuclease produced only 5 different ribotypes with sixteen clinical *V. cholerae* isolates, compared to 10 with traditional

ribotyping. Automated ribotyping using *BglI* produced 7 ribotypes compared to 10 with traditional ribotyping. The lower discrimination shown by the *Riboprinter system* was caused mainly by an inability to differentiate closely related fragments due to lower resolution and electrophoreses conditions, a parameter that cannot be changed in an automated system. This system therefore is not adequate for the taxonomic identification and classification of *V. cholerae* (Dalsgaard *et al*., 1999.). Bik *et al*. (1996.) described a novel *V. cholerae* IS element (IS1004) and used it in a probe based RFLP analysis to differentiate between *V. cholerae* strains. DNA restriction patterns of O1 strains showed close similarity, but biotypes Classical and El Tor could be distinguished. Several Non-O1 strains gave heterogeneous fingerprints indicating that this method could also be applicable for Non-O1 strains.

2.8.2.2.2 PCR-RFLP

In PCR-RFLP selected sequences are amplified with PCR and digested with restriction enzymes before fragment detection takes place. Yam *et al.* (1991*)* used the technique of PCR-RFLP analysis to study the molecular epidemiology of *V. cholerae*. Strains of *V. cholerae* El Tor serotype Inaba isolated in 1989 from a limited cholera outbreak in a Vietnamese refugee camp were compared with several indigenous and exogenous strains isolated during the same period. RFLP of the enterotoxin gene was used as the epidemiological marker. They found that all strains isolated from the outbreak were indistinguishable, but that they were distinct from isolates isolated in Hong Kong previous to the outbreak.

2.8.2.3 Sequencing of specific genes

2.8.2.3.1 Single-locus sequence typing (SLST)

Olsvik *et al*. (1993) used automated sequencing of PCR generated amplicons to identify three types of cholera toxin sub-unit B in *V. cholerae* O1 strains. DNA sequencing of cholera toxin sub-unit B structural genes from 45 *V. cholerae* O1 strains isolated in 29 countries over a period of 70 years were determined by automated sequencing of PCR generated amplicons. Three types of cholera toxin sub-unit B were identified using strains originating from various countries. Due to the low discrimination of single locus sequence typing, this system will probably not have wide application.

2.8.2.3.2 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST), as originally described (Maiden et al., 1998), involves the determining of the nucleotide sequences of a series of housekeeping genes. MLST provides a balance between sequence based resolution, informativeness, and technical feasibility and has been applied for various bacteria, including *Vibrio cholerae* (Byun *et al.*, 1999; Thompson *et al*., 2004). In a study by Kotetishvili *et al.* (2003) where twenty-two *Vibrio cholerae* isolates (O1, O139, and non-toxigenic strains) were characterized by MLST and pulsed field gel electrophoreses (PFGE), it was found that MLST had the better discriminatory ability of the two techniques. Furthermore MLST data is unambiguously comparable among laboratories as it is based on nucleotide sequences.

2.8.2.4 Selective Restriction Fragment Amplification

AFLP™ was developed by KeyGene International (Wageningen, The Netherlands) as a universal DNA fingerprinting method with application in a large variety of fields (Vos *et al.*, 1995). This technique has been shown to be able to analyze any kind of DNA, regardless of its source, composition or complexity (Janssen *at al*., 1996). Advantages of the technique is that no prior sequence knowledge is required, it is robust and reliable due to the stringent reaction conditions used for the PCR amplification, and it is sensitive due to the incorporated PCR (Vos *et al.*, 1995). The AFLP technique can be summarized in three steps: I) Digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site specific adapters to all restriction fragments, II) Pre-amplification and Selective amplification of a subset of the fragments, with PCR primers directed at the corresponding restriction enzyme site and adapter sequences III) Electrophoretic separations of amplicons on a gel matrix, followed by visualisation of the banding pattern.

AFLP has been widely used to type clinical as well as, to a lesser extent, environmental *Vibrio cholerae* strains, as the ability to discriminate closely related strains makes it an invaluable tool. Jiang *et al*. (2000 a, 2000 b) used this technique to determine the genetic diversity of clinical and environmental *Vibrio cholerae* isolates, obtained during and between epidemics over the last twenty years. Two sets of primer combinations were tested: I) *HindIII* and *TaqI*, this combination was able to distinguish between O1 and non-O1 isolates, but was unable to distinguish between O1 and O139 isolates. II) *ApaI* and *TaqI* which was able to distinguish between O1 and O139 isolates. These results

confirm that O1 and O139 strains are very closely related. The researchers suggested that a single clone of pathogenic *Vibrio cholerae* appeared to be responsible for many cases of cholera in Asia, Africa and Latin America during the seventh pandemic. These conclusions were strengthened by a study done by Lan and Reeves (2002). A collection of 45 seventh pandemic isolates of *Vibrio cholerae* sampled over a 33-year period was analyzed by AFLP. They found that this technique gave far better results than Ribotyping, the previously preferred technique for strain differentiation, within the pandemic clone. AFLP grouped most of the isolates into two clusters; cluster 1 containing mostly isolates from the 1960's and 1970's, while cluster 2 contained isolates from the 1980's and 1990's. This data suggests a temporal pattern of change within the pandemic clone. AFLP has also been used with great success in the typing of *V. cholerae* strains, the ability to alter the level of discrimination by changing the selective parameters providing researchers with a tool that can be used for global epidemiological as well as regional environmental studies. Jiang *et al*. (2000 a) used AFLP to characterize the temporal and spatial genetic diversity of environmental *V. cholerae* isolates obtained from Chesapeake Bay. Sixty-seven non-O1 *Vibrio cholerae* isolates were isolated from Chesapeake Bay and AFLP was used to characterize the temporal and spatial genetic diversity of these isolates. Clusters reflected the sampling time, with no correlation being observed between geographical source and clustering. It was suggested that certain environmental conditions (water temperature etc.) might select for specific *V. cholerae* strains, which may dominate the population until another strain is selected for by changing conditions. Thompson *et al* found environmental *Vibrio cholerae* strains to be genetically diverse using AFLP. The persistence of some strains with highly related

genomes over several years, in different geographical regions suggesting that some strains can adapt successfully to changing environmental conditions (Thompson *et al.*, 2003).

AFLP data generated by researchers have given new insight into the origins, routes of spread and population dynamics of *Vibrio cholerae*. Studying environmental populations with AFLP might further our understanding of *V. cholerae* epidemiology, especially with reference to the study area, thus enabling the implementation of effective water management strategies.

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