Critical review of PCR protocols for the detection of *Vibrio cholerae* in wastewater: A collaborative, inter-laboratory blinded proficiency testing study

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Executive summary

With countless published reports showing that the conventional culture approach to enumerate *Vibrio cholerae* (*V. cholerae* [cholera-causing disease]) is labour intensive, less sensitive, complex with a thorough competent analysis needed, the advent of polymerase chain reaction (PCR) compelled a need for an alternative approach. Although research institutions have developed PCR protocols that carry accreditations, studies have shown that multicentre comparison of diagnostic detection tools is necessary for validation purposes and to increase diagnostic sensitivity and specificity. Therefore the present study investigated the validity of an accredited PCR method, in comparison with previously published PCR protocols; to routinely monitor the occurrence and distribution of *V. cholerae* in environmental wastewater samples.

Twelve wastewater treatment plants (WWTP) situated in the Gauteng province, South Africa, were selected for this study. Two laboratories were involved and included; ❶ Water and Health Research Centre (WHRC) from the University of Johannesburg (UJ) and ❷ an Independent Accredited Laboratory (IAL [commercial, ISO 17025 accredited water testing laboratory]). An IAL conducted one PCR protocol that carried an accreditation, while the WHRC laboratory conducted three PCR protocols; two previously published PCR protocols and a real-time PCR commercial kit. All the PCR assays included genes specific for *V. cholerae* as species except for the commercial kit, which was specific for all the *Vibrio* species. Through evaluating the four PCR assays suitability and efficiency in wastewater samples, the occurrence and distribution of *V. cholerae* and *Vibrio* species were determined.

A total of 136 wastewater samples sampled by a laboratory personnel (from IAL) that included equal parts of raw influents and final (treated) effluents and transported to IAL and WHRC laboratories. Samples were received, processed and shared between the two laboratories to generate comparison of results. From a total of 136 wastewater samples processed at IAL; 68 DNA, 28 APW pre-enrichments and 40 water samples were shared with WHRC. From this, 40 DNA samples processed (from water samples) at WHRC was shared with IAL.

All the experiments included pre-enrichment of 100 ml filtered environmental wastewater samples in 100 ml alkaline peptone water (APW) for 16 hours at 37 °C prior to genomic DNA extraction and PCR analysis. Two DNA extraction protocols were employed, namely the Instagene™ Matrix (commercialized kit) from IAL and an in-house optimized guanidinium thiocyanate (GuSCN) method at WHRC. Extracted genomic DNA was tested for the

amplification of specific PCR products. An accredited, high resolution melt real-time PCR (HRM real-time PCR; IAL) specifically amplified the *ompW* (*V. cholerae* species-specific) and *ctxAB* (cholera toxin) genes in a duplex assay. The remaining three PCR's were conducted at the WHRC. A Taqman real time PCR was carried in two duplex assays; the first duplex targeted the *ctxA* (cholera toxin) and *hlyA* (*V. cholerae* species) and the second duplex targeted O1-*rfb* (*V. cholerae* O1) and O139-*rfb* (*V. cholerae* O139), with *gfp* gene complementing each duplex as process internal amplification control (IAC). Conventional PCR was carried in a quadriplex assay, targeting *V. cholerae* species-specific (*V.c SodB*) gene, serogroup-specific *V. cholerae* O1 and *V. cholerae* O139 *rfb* genes, and cholera toxin gene (*ctxA*). A real-time commercial PCR kit was performed in a singleplex assay, targeting *Vibrio* species gene.

The results of this study showed that none of the raw influent or treated effluent samples tested positive for toxicity (*ctxA*/*ctxAB* genes) or the specific *V. cholerae* serogroup O1/O139*rfb* genes irrespective of the PCR assay used. However, variability in the positive detection of *V. cholerae* genes (*hlyA*, *sodB* and *ompW*) as species (including *Vibrio* spp. gene) was observed. The overall results for the detection of non-toxigenic *V. cholerae* with HRM real-time PCR in wastewater samples were; 72 (52.9 %) positive in 136 wastewater samples, 38 (55.9 %) positive in 68 DNA samples, 18 (64.3 %) positive in 28 APW enrichments and 16 (40 %) positive in 40 water samples. With Taqman real-time PCR, the detection of *V. cholerae* was; 75 (55.1 %) positive in 136 wastewater samples, 40 (58.8 %) positive in 68 DNA samples, 19 (67.3 %) positive in 28 APW enrichment samples and 16 (40 %) positive in 40 water samples. With conventional PCR, the results for the detection of *V. cholerae* was; 37 (27.2 %) positive in 136 wastewater samples, 15 (22.1 %) in 68 DNA samples, 15 (53.6 %) positive in 28 APW enrichment and 7 (20 %) positive in 40 water samples. The results for the detection of *Vibrio* species with commercial kit in wastewater samples was; 37 (34.6 %) positive in 136 wastewater samples, 11 (16.2 %) positive in 68 DNA samples, 21 (75 %) positive in 28 APW enrichment and 15 (37.5 %) positive in 40 water samples. The highest detection rate of *V. cholerae* and *Vibrio* species when combinations of the four PCR's were made was obtained with a combination of HRM real time PCR and Taqman real-time PCR, with concurrent detection of 64 (47.1 %) samples tested positive for *V. cholerae*.

The results from this study further showed that Taqman and Evagreen HRM real-time PCR assays, combined with a pre-enrichment step, have the potential to be employed in a routine laboratory setup for the active monitoring of the occurrence and distribution of toxigenic *V. cholerae* in wastewater treatment facilities. The results also indicated the importance of an

appropriate DNA extraction method to ensure assay sensitivity and limit PCR inhibition. The inclusion of an IAC in Evagreen HRM real-time PCR should be considered to ensure that no false negative results are reported. This study also addressed the need for collaborative, inter-laboratory proficiency testing schemes to validate PCR detection assays for routine use in water quality testing.

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List of Abbreviations

°C	Degrees celcius
ACF	Accessory Colonization Factor
AE	Elution buffer
APW	Alkaline Peptone Water broth
ATCC	American Type Culture Collection
BOD	Biological Oxygen Demand
CFR	Case Fatality Rate
CFU	Colony Forming Unit
Cl	Chlorine
COD	Chemical Oxygen Demand
ctxA	Cholera Toxin gene
Ct	Threshold Cycle
CT	Cholera Toxin
$CTX^{class}\Phi$	CTX classical
$CTX^{ET}\Phi$	CTX El Tor
$CTX^{calc}\Phi$	CTX Calcutta
DNA	Deoxyribonucleic acid
DPW	Department of Public Works
DVC	Direct Viable Count
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EDTA	Ethylene Diamine Tetraacetate
EPA	Environmental Protection Agency
EPS	Extracellular Polymeric Substance
FC	flow cytometry
gfp	Green fluorescence protein gene
GuSCN	Guanidium Thiocyanate
HCl	Hydrochloric Acid
HRM	High Resolution Melt
IAC	Internal Amplification Control
IAL	Independent Accredited Laboratory
ISO	International Organization for Standardization
mg/l	Milligram per litre
mS/m	Milli-Siemens per Meter
NAG	Non-agglutinating
NASBA	Nucleic Acid Sequence-based Amplification
NCBI	National Centre for Biotechnology Information
NHLS	National Health Laboratory Services
NCTC	National Collection of Type Cultures
nm	Nanometre
NTC	None Template Control

TCBS	Thiosulfate Citrate Bile salts Sucrose
TCP	Toxin Coregulated Pilus
V. cholerae	*Vibrio cholerae*
PCR	Polymerase Chain Reaction
PTC	Positive DNA Template Control
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Tm	Melting Temperature
UJ	University of Johannesburg
UV	Ultraviolet
VBNC	Viable but non-culturable
VPI	'*Vibrio* Pathogenicity Island'
WHO	World Health Organization
WWTP	Wastewater Treatment Plant
WHRC	Water and Health Research Centre

CHAPTER 1

INTRODUCTION AND LITERATUE REVIEW

1 Introduction

Studies indicate that wastewater remains a significant health hazard with a principal area of concern the high content of microbiological pathogens that include bacteria, viruses and helminths, which are readily transmitted through oral-fecal route (WHO, 2006; Ismail et al., 2011). The epidemiological implications of this could be that the microbiological contamination of wastewater continues to contribute to the spread of diarrheal diseases such as cholera (Curtis, 1996). This disease (cholera) is of epidemiologically importance for its ability to reach epidemic proportions (DWAF, 2002). The epidemiology of cholera has been dominated by its tendency to spread throughout the world in pandemics (Table 1.1) (Hunter, 1997). Therefore the inability to halt cholera epidemics is facilitated by infected individuals who are not ill, but are carrying cholera causing *V. cholerae* bacteria, in their faeces (DWAF, 2002). These individuals, epidemiologically regarded as asymptomatic carriers, continue the cholera cycle by shedding the bacteria into aquatic environments through inadequately treated wastewater discharge. For this reason, active monitoring of *V. cholerae* in wastewater treatment facilities is of paramount importance to curb cholera cases as well as to ascertain the potential health impacts of wastewater treatment plants.

The conventional culture method to detect *V. cholerae* in source waters includes filtration of water samples (usually 100 ml) with subsequent enrichment of the filtrate in Alkaline Peptone Water (APW) broth. Sub-culturing of enrichment broth onto Thiosulfate Citrate Bile salts Sucrose (TCBS) agar is followed by confirmation tests, such as verification of the presumptive isolates through serological assessment to identify the *V. cholerae* O1 serotype antigens (Lesmana et al., 1997). A serological confirmation involves the reaction of microorganism (antigen) with its corresponding antibody (Difco™ *Vibrio cholerae* antisera, 2003). The conventional culture based method alone is far too expensive and complicated and as a result, a competent analyst is needed to successfully conduct the procedure. Furthermore, the method is less sensitive and very tedious, with a lengthy period of days needed to complete the detection procedure (Ashbolt, 2003; Lijima et al., 2004; Kim et al., 2011). The advent of polymerase chain reaction (PCR) assays to alternatively succeed the conventional approach has led to numerous *V. cholerae* PCR assays been

formulated, with most of them showing reproducible sensitivities and specificities (Gubula & Proll, 2006; Gubula, 2006; Blackstone et al., 2007; Huang et al., 2009; Girones et al., 2010). However due to their (i.e. PCR assays) lacking in standardization, the conventional culture method remains the gold standard as a reliable diagnostic detection tool for the enumeration of *V. cholerae* from the environment (DWAF, 2002).

To date, there is no standardized PCR method for the detection of *V. cholerae*. This is mainly due to the fact that all the existing PCR assays are in-house optimized protocols using different DNA extraction methods, primer and/or probe sets, reaction conditions and methods of detection (Apfalter et al., 2001). However, the introduction of blinded inter-laboratory comparison of diagnostic detection tools has been the motivation to find the solution of standardizing PCR assays. On the other hand, commercial, ISO 17025 accredited water testing laboratory institutions have optimized and adopted PCR protocols that they use for routine detection in active monitoring setting. Although these institutions have assigned these PCR assays as 'accredited', they lack appropriate validity in terms of relevant controls that needs to be included. The universal prerequisite for a non-commercial PCR assay to be adopted as a standardized method in scientific literature requires the method to be non-proprietary and validated, through collaborative trials in the form of blinded proficiency tests, in different accredited scientific laboratories (Hoorfar et al., 2003). In this instance, PCR assays which include process internal controls in their protocol is needed to validate the assurance that the results obtained are true-positive, with certainty that no inhibition is encountered (Rodriguez-Lazaro et al., 2004). Furthermore, inclusion of blinded inter-laboratory comparison of PCR assays in collaborative trials will enable the coherent basis of comparison of results as well as to motivate the reliance of such results (Pellett et al., 1999).

With this procedure, the PCR assay results that are closest could be used as a reference standard for subsequent standardization experiments (Flamand et al., 2008). Therefore it is of paramount importance that for any PCR assay that is to be published, or already published, the validity and reproducibility of the PCR be tested in variety of sample sources and in a collaborative, inter-laboratory proficiency trial experiments in order to ascertain their validity.

2 Literature review

Wastewater is a representation of contaminants that includes a range of pathogens such as bacteria, viruses, parasites and toxic chemicals (heavy metals and organic chemicals) from domestic, agricultural and industrial sources (Corcoran et al., 2010). The estimated proportion of wastewater from human settlements and industrial sources that has been discharged untreated into the environment is said to be 80 % worldwide (The Post 2015 Water Thematic Consultation report, 2013). The need to treat wastewater prior to discharge into rivers and streams is of paramount importance for downstream activities that includes; fishing, swimming and drinking (EPA, 2004). The health impacts associated with contaminated wastewater include common diseases such as swimmer's itch, gastro-enteritis, dermatitis, viral hepatitis, wound infections, cholera, typhoid fever, and dysentery, which have been linked with bathing in contaminated recreational water or consumption of contaminated sea food or water (WHO, 2005). Of the aforementioned diseases, cholera remains a global threat as it is the only waterborne disease that could reach epidemic proportions (DWAF, 2002).

In 1855, John Snow indicated the relationship between contaminated sewage water and cholera (Okoh et al., 2007). In South Africa, the recent outbreak of typhoid fever experienced in the Mpumalanga province was shown to have originated in the town water supply, which was suspected to be contamination by human faeces (Momba et al., 2006). The cholera outbreak reported in Delmas (i.e. town of Mpumalanga province) was also indicated to be due to the declining state of municipal wastewater and sewage treatment infrastructure (Mema, 2010). Some of the factors that were identified to have contributed to this outbreak were; 1) poor plant design, 2) poor enforcement of environmental law, 3) lack of skilled personnel and 4) poor operation and maintenance of WWTP. The relationship between typhoid fever with consumption of municipal wastewater was also indicated in central Asia (Mermin et al., 1999). Cases of diarrhea linked with drinking municipal water were also indicated by Hunter (2003). In South Africa, a diarrheal outbreak was reported in the Mhlathuzi River, KwaZulu Natal province in July 2000, which was followed by cholera epidemic (Bezuidenhout et al., 2001). The introduction of cholera in South Africa was reported to have been facilitated by hot humid summer seaports, overcrowded communities, with sub-standard environmental sanitation and scanty, restricted and unprotected water supplies in the KwaZulu Natal province (Mugero & Hoque, 2001). The epidemic spread to

other provinces including Mpumalanga, Eastern Cape, Gauteng and North West (Hoque & Worku, 2005). Samie et al. (2009) have also reported cases of cholera in Gauteng province. A summary of cholera cases which affected South African provinces in 2001, is shown in Table 1.1 (Mugero & Hoque, 2001).

Table 1.1 Cholera cases reported in nine South African provinces indicated with their case fatality rates (CFR) (adopted from Meguro & Hoque, 2001)

Province	Date	Cases	Deaths	CRF (%)
KwaZulu Natal	4/1/2001	80,387	168	0.2
Limpopo	4/2/2001	741	9	1.21
Mpumalanga	3/21/2001	79	2	2.53
Gauteng	3/29/2001	49	3	6.12
North West	3/12/2001	6	0	0
Wastern Cape	3/26/2001	1	0	0
Free State	3/26/2001	1	0	0
Western Cape	4/2/2001	1	0	0
Northern Cape	3/26/2001	0	0	0

Cholera is transmitted via the fecal-oral route and is prevalent in developing regions where there is a problem with food or water supply, overcrowding and inadequate sanitation (Ismail et al., 2011). Contaminated wastewater coupled with poor sanitation and lack of personal hygiene, has been the mainstay in continuing the spread of cholera worldwide (DWAF, 2002). In 2008-2009, a cholera outbreak occurred in Zimbabwe and took a sub-regional dimension, spreading quickly in South Africa resulting in 3907 cases and 22 fatalities (Madoroba & Momba, 2010). The ability to halt cholera cases is implicated on implementation of the best intervention practices that includes; proper development and maintenance of wastewater treatment plants as well as keeping contaminated wastewater from water, food and environment (The Post 2015 Water Thematic Consultation report, 2013). Although implementation of these interventions could be a miracle expectation in a short space of time, the one intervention can surely be to rely on the effectiveness of WWTP to prevent cholera into reaching the environment.

In 2010, it was indicated that only 23 out of 900 WWTP in South Africa met the standard set by DAWF for effective functioning of WWTP (UASA Water Security Seminar, 2010). Moreover, older large urban areas along the coast have collecting systems in place for municipal wastewater with deteriorating infrastructure that result in regular seepage and spillage. These problems are also encountered in the rapidly expanding informal settlements with difficulty in finding supply of effective collection system. These factors are an indication of poor quality effluents with attendant negative consequences on the receiving watersheds (Osode & Okoh, 2009). The removal of faecal contaminants in wastewater is facilitated by proper functioning of various compartments in WWTP which includes; preliminary, primary, secondary and tertiary treatment (DWAF, 2004).

2.1 Wastewater treatment plants

2.1.1 Operational wastewater treatment process

Several efforts have been pursued to control environmental pollution which include monitoring the effluent's physico-chemical parameters as well as microbiological quality of the final effluent in municipal treatment facilities (Igbinosa & Okoh, 2008; Igbinosa & Okoh, 2009). The physico-chemical parameters measured include pH, temperature, electrical conductivity, salinity, turbidity, total dissolved solids, dissolved oxygen, chemical oxygen demand, nitrate, nitrite and orthophosphate levels (Igbinosa & Okoh, 2009).

General and special standards exists in South Africa for wastewater pollutant limits applicable to the discharge of wastewater into water resources and is governed by the National Water Act, 1998 (Department of Public Work, 2012). For microbiological quality, removal of pathogenic microorganisms is rendered to achieve objectives which include: 1) the protection of quality of the water sources; 2) the reduction of costs of drinking water treatment; and 3) the control or prevention/elimination of waterborne diseases (Dungeni et al., 2010). Microbial pathogens such as bacteria, viruses, protozoa, fungi and helminthes enter the sewer system from domestic wastewater and industrial wastewaters through inflow and infiltration (Momba, 2010). Apart from the performance of the sewer plant, the production process of the sewage treatment plant is required to comply with the Occupational Health and Safety Act No 85 of 1993 (Department of Public Works, 2012). For assessment of the effectiveness of the wastewater treatment plant, monitoring systems must be in place that will determine whether the treatment system effectively

remove pathogens followed by subsequent production of quality effluent that meets the required standard specifications (Dungeni et al., 2010) (Table 1.2). The Department of Water Affairs (DWA) has introduced an incentive-driven approach called the Green drop certificate, which measures the performance of wastewater treatment works nationwide. It is designed in a manner that categorizes the municipal sewage treatment works based on the Green Drop status were; a score is allocated to best performing treatment plants that meets the standard requirements set by the Department of Water Affairs (DWA, 2009).

Table 1.2 Water quality specifications as amended by the SA water Act No 36 of 1998 (DWAF, 1998)

Substance/Parameter	General Limit	Special Limit
Faecal Coliforms (per 100 ml)	1000	0
Chemical Oxygen Demand (mg/l)	75 after removal of algae	30 after removal of algae
pH	5.5 - 9.5	5.5 - 7.5
Chlorine as free Chlorine (mg/l)	0.25	0
Suspended Solids (mg/l)	25	10
Electrical Conductivity (mS/m)	70 mS/m and max of 150 mS/m	50 mS/m and max of mS/m

For effective removal of microbiological contaminants in sewage, four operation units within the WWTP are needed which include; preliminary, primary, secondary and advanced or tertiary treatment and explained briefly below according to Wells, (2005) and Okoh et al., (2007). A general overview of WWTP is illustrated in Figure 1.1.

2.1.2 Four operation units of wastewater treatment plant process

2.1.2.1 Preliminary treatment

Wastewater enters the WWTP through preliminary treatment, which employ screens and grits inside the holding chambers inclined towards the flow of wastewater for the removal of large floating objects such as rags, cans, bottles and sticks that may clog the process downstream.

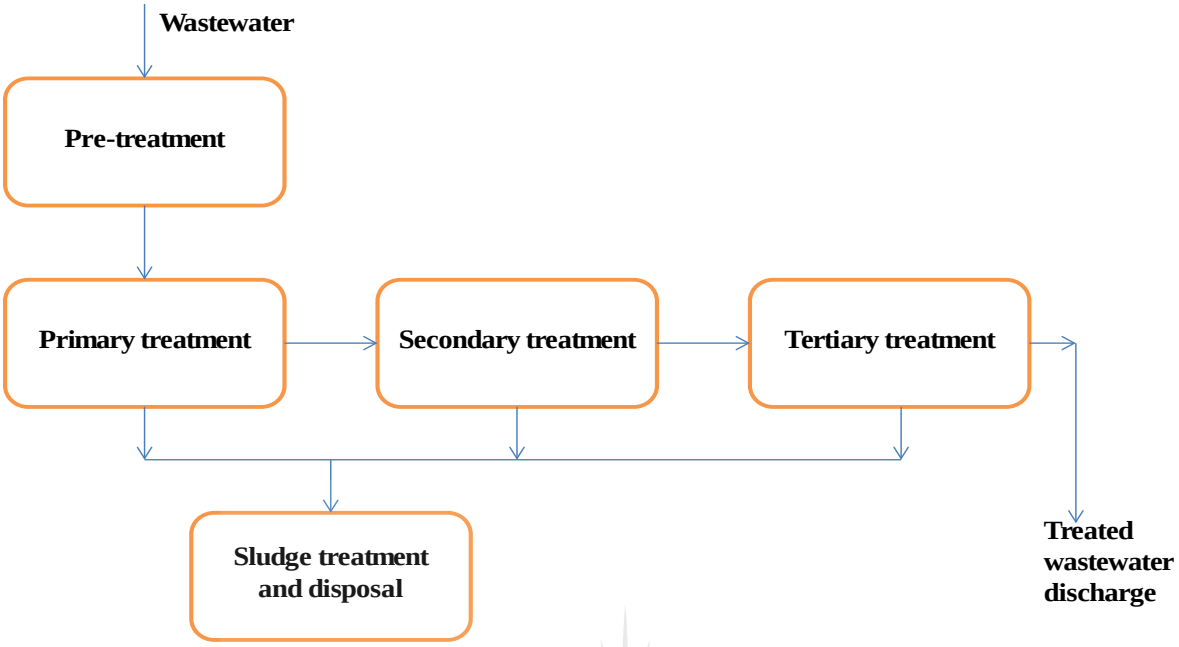

Figure 1.1 Overview of wastewater treatment process indicating four unit operations (source: Armenante, www.njit.edu)

2.1.2.2 Primary treatment

Removal of suspended solids and grease is carried out in the primary treatment. This stage involves sedimentation of settleable solids and skimming of floating oil and grease. Sediments of settleable solid and organic matter can be removed from the treatment process by suction pumps, bucket dredges or manually. For skimming of floating oil and grease a scum board is required to prevent floating material from passing over the outlet weir. In some plants, preliminary and primary treatments are incorporated into one unit operation as primary treatment. The removal of up to 40% of the Biological Oxygen Demand (BOD) can be attained, with suspended organic material passing through to the next unit. For bacterial removal, an efficiency of 40 to 70% can be eliminated (Veenstra, 1996).

2.1.2.3 Secondary treatment

In secondary treatment, dissolved organic matter (and soluble organic matter) not removed in primary treatment is further removed up to 90% efficiency. This is an aerobic process that utilizes

microorganisms to oxidize the organic matter as their food supply for growth, coupled with aeration of air or alternatively agitation for oxygen transfer. Also organic nitrogen-containing material is removed by converting ammonia nitrogen to nitrite. Numerous biological process technologies exist but the most commonly used include trickle filters or activated sludge. Trickle filters employ a bed medium on which water flows over and microorganisms decompose organic matter into a biofilm-like structure of microbial biomass. Microorganisms such as bacteria, protozoa, algae and fungi constitute the biofilm (known as zoogleal film) formed by attachment and proliferation of the bacteria on the surface of an object. Attachment is facilitated by a biofilm slimy layer created by exopolysaccharide (EPS) secreted by bacteria for entrapment of microorganisms. Activated sludge involves the use of bacteria-rich slurry mixed with primary effluent, with vigorous aeration for oxidation of organic material and formation of bacterial flocs. With this process (activated sludge), up to 90% of BOD can be removed. With activated sludge and trickle filter, up to 99% of pathogenic microorganisms can be removed. In secondary treatment, sufficient retention time is needed to allow high degree of pathogen removal (Veenstra, 1996; WHO, 1997).

2.1.2.4 Tertiary treatment

The final step, tertiary or advanced treatment, facilitates the removal of suspended and dissolved substances remaining after secondary treatment. This process usually involves physicochemical processes such as coagulation, filtration, activated carbon and chlorination. Removal of ammonia, as well as microorganisms is facilitated by these physical, chemical and biological processes. The chemical process includes the disinfection process to destroy pathogens. The most commonly used disinfectants are chlorine, ozone and ultraviolet radiation (Veenstra, 1996).

2.1.2.5 Quaternary treatment

Quaternary treatment process, sometimes referred to advanced treatment (like tertiary treatment), is usually required for more advanced, high level treatment (such as for recreation or recreation reuse) which uses special advanced technologies such as membrane processes and ion exchange. The membrane process employs variety of filtration processes with varying exclusion sizes including ultrafiltration (2-50nm), nanofiltration (<2nm) and reverse osmosis (<1nm) depending on the nature of material to be removed. This can range from *Giardia* and bacteria, suspended

solids, viruses and proteins, organics and pesticides, to metals, dissolved solids, nitrates and radionuclides. Ion exchange is highly effective for special removal of cations (such as calcium, magnesium and iron) and anions (nitrate). However different designs of treatment processes are found, some can incorporate tertiary and quaternary treatment into one unit (i.e. tertiary treatment), while in other processes they are separated. Advanced treatment is normally employed for high quality effluent required for reclamation of groundwater recharge or for discharge to recreational waters (Veenstra, 1996; WHO, 1997).

2.1.2.6 Disinfection

Disinfection is classically referred to as the partial destruction of pathogens to acceptable limits, as opposed to sterilization for the entire removal or destruction of all pathogens present. Therefore since sterilization cannot be employed in the wastewater treatment process due to large quantities of water (as compared to laboratory scale), a more feasible process, disinfection, is used. The disinfection capability of chlorine in wastewater is used in one of its different forms including gaseous chlorine, sodium hypochlorite solution, calcium hypochlorite and bromium chloride. The persistence of chlorine residuals in treated wastewater is normally dechlorinated with chemicals such as sulphur dioxide, sodium bisulphites, sodium metasulfite and activated carbon to protect aquatic life and the environment. Although the use of chlorine disinfection in wastewater is advantageous, free residual chlorine can be toxic to downstream aquatic species even at low concentration. However it (i.e. chlorine disinfection) is preferred due to it's; 1) cost-effectiveness, 2) ability to prolong disinfection, 3) reliability to and effectiveness against a wide spectrum of microorganism and 4) ability to allow flexible dosing (Solomon et al., 1998). Ozone disinfection is also a powerful disinfectant but requires high doses and is more expensive than chlorine. Ultraviolet disinfection is more advantageous than chemical disinfection in that little supervision (and maintenance) is needed and requires only a little contact time (with wastewater) with no toxic by-products formed. Its main limitation is that turbid water (dissolved and suspended solids) renders low disinfection performance (Solomon et al., 1998; National Drinking Water Clearinghouse, 2000).

2.3 Survival of bacterial pathogens in WWTP

Survival of bacteria (including pathogens) in sewage treatment plants will depend on the design of the plant, the availability of the technologies employed as well as the state of the plant. The quality of the treatment methods applied is of primary importance in eliminating or reducing microbiological pollutants (Paluszak et al., 2003). Hendricks & Pool (2012) have reported the effectiveness of new technologies such as membrane bioreactors in WWTP. These authors indicated that membrane bioreactor technology was effective in removing microbiological pollutants in the treatment plant as compared to older technologies that employs only disinfection (chlorination and UV radiation). Moreover Dungeni & Momba (2010) reported rapid sand filtration as a means of high quality effluent production. The quality of the sewage effluent influences the disinfection efficiency. Poor quality effluent has suspended solids that the bacteria use to entrap and shield against disinfection (Dungeni et al., 2010). The bacteria, when entrapped in suspended particles, can remain resistant to disinfection even at high residual chlorine disinfection treatment (Dungeni et al., 2010).The effect of physico-chemical parameters, which include pH, temperature, turbidity and conductivity, have also been reported on the survival of bacteria in sewage treated effluents (Salem et al., 2011).

Studies by Paluszak et al. (2003) have shown that bacteria frequently survive sewage treatment during winter seasons regardless of the type of technologies used in the sewage treatment facility. However this could not be explained as to how these microbes survive. Other studies have reported the decrease in pH as a devitalizing effect in microorganisms (Plachá et al., 2001). Kantachote et al. (2009) have indicated higher pH values (8.5) as a need to inactivate organisms when *E. coli* was used as a model. Rojas & Hazen, (1989) reported that competition of high microbial load in the effluents can facilitate the survival of bacteria against disinfection. Furthermore these authors indicate that a large population of microbes exposed to disinfection may lead to dead cells, partially dead and/or high resistant phenotypes thereby forming a layer around viable cells. This can be further reasoned that low efficiency of bacterial removal by the treatment process, ultimately lead to low disinfection efficiency. A systematic study by Rockabrand et al. (1999) has shown that chlorination does not effectively kill the cells but rather elicit nutrient starvation and a reversible nonculturable state. It was indicated that chlorine disinfection simply injures *V. cholerae* and that resuscitation of injured cells is of importance in the dissemination to the environment. In studies

conducted in South Africa, Samie et al. (2009) have reported the survival of *V. cholerae* following chlorine disinfection in WWTP plants in the Mpumalanga province. Factors that were identified to cause the survival of this pathogen in the final effluent included; 1) irregular recharge or replacement of disinfection agent and 2) the dosage of chlorine facilitated based on experience and not on real situation such as the knowledge of the water flow in the plant.

2.4 Biofilm formation

Roszac & Colwell (1987) have defined survival of microorganisms as the maintenance of viability under adverse conditions. The ability of prokaryotes to persist throughout the biosphere is based on their outstanding metabolic versatility and phenotypic plasticity (Abraham, 2011). Water properties, such as temperature, salinity, pH, sunlight, oxygen content and nutrient availability, can change dramatically, temporally or spatially (Milton, 2006). The persistence of microorganisms in aquatic ecosystems is facilitated by adhesion to organic or inorganic surfaces, followed by binary fission exopolymer production to develop a matrix-enclosed sessile mode called biofilm. Cells in biofilms can either be composed of pure culture or comprise mixed microbial species (Huq et al., 2008). Bacterial cells in biofilms are rarely planktonic in aquatic environments, instead are associated with variety of surfaces that can include non-cellular materials such as mineral crystals, corrosion particles, clay or silt particles, blood components as well as living tissues, in medical devices, industrial or potable water piping systems, with attachment helping the bacteria from being swept away (Vu et al., 2009). Donlan (2002) has indicated that biofilm associated cells can be differentiated from their suspended counterparts by the production of extracellular polymeric substance (EPS) matrix, reduced growth rates, and up- and down-regulation of specific genes. Cells embedded in the matrix communicate with each other (cell-to-cell communication), with coordinated group behavior mediated by a process called quorum sensing (Coenye & Nelis, 2010). Quorum-sensing in *V. cholerae* is known to be a regulatory cascade for virulence genes and function by fine-tuning the expression of virulence factors to that of the cell population density (Milton, 2006). Bacterial cells in biofilm are protected from adverse environmental conditions and chemical antibacterial agents (Leclerc, 2003). The adherent cells in biofilm express a different transcriptome when compared to planktonic cells (i.e. free-living cells) (Nagant et al., 2010). Surface attachment following biofilm formation play an important role in the environmental survival of *V. cholerae* because; 1) nutrients tend to be

concentrated on surfaces, 2) biofilm formation improves bacterial survival during osmotic and chemical stress, and 3) a single clump of biofilm-associated cells can reach a cell density needed to cause infection, with lower cell density attained with planktonic cells (Van Dellen & Watnick, 2006; Huq et al., 2008). Bioflm formation in *V. cholerae* was also reported in copepods, were a carapace of single copepods was shown to have the ability to harbour as many as 10^4 *V. cholerae* . The concentration of cells in aquatic animal and plant species decrease the volume of water required to deliver an infectious dose (Van Dellen & Watnick 2006). Attachment, survival and proliferation of *V. cholerae* on the surface of copepods have also been reported in laboratory experiments (Huq et al., 1990).

Attachment to aquatic species including phytoplankton, zooplankton, fish, molluscs and crustaceans, insects, as well as sediments, serve as a survival strategy for *V. cholerae* to enter a dormant state known as viable but non-culturable (VBNC) (Colwell et al., 1996; Borroto, 1997; Halpern et al., 2006; Fong et al., 2010). A study by Gil et al. (2004) has shown that attachment of *V. cholerae* to zooplankton was shown to be higher as compared to other bacterial taxa such as γ-proteobacteria and photobacterium. The author suggested that the strong association of *V. cholerae* to copepods could be of commensal indicated that Biofilm-growing cells thrive in unfavorable micro-environmental conditions such as nutrient and oxygen depletion, pH and ionic strength variation, and may undergo transformation into slow-metabolism forms including VBNC state (Zandri et al., 2010).

2.5 Viable but nonculturable cells

The ability of pathogens such as *V. cholerae* to persist in the aquatic milieu for extended periods is modulated by its morphological change (coccoid shape) to enter a dormant VBNC state (Alam et al., 2007). Zandri et al. (2012) explain the VBNC state as a transient dormancy condition for stress adaptation, associated with cell dwarfing and failure to grow on laboratory culture media. *V. cholerae* preferably grow in low salinity environments when the water temperature is relatively high, when organic nutrients are high enough concentrations, to compensate to some degree, lack of salt (Asakura et al., 2006). When these conditions becomes unfavourable, the bacteria enters a dormancy state (VBNC) that enables the bacteria to persist in aquatic environment as a means of survival by, in some instances, attachment to aquatic planktonic species in response to low temperatures and reduced nutrient concentrations (Binzstein et al., 2004). Cells in a VBNC state

demonstrate metabolic activity; maintain virulence features and antibiotic resistance traits, are capable of gene expression and exchange, and capable to resume division when favourable environmental conditions are restored (Lleo et al., 2005). The survival of bacteria in drinking water, which represent unfavourable environment with limited nutrients, causes a dramatic decrease in bacterial plate count but remain viable when analysed by sensitive viability techniques such as direct viable count (DVC) involving fluorescence-labelled monoclonal antibodies (Byrd et al., 1991; Colwell et al., 1996).

2.6 *V. cholerae* and its mode of infection

V. cholerae serogroups that agglutinate with O1 and O139 antiserum are the aetiological agents of cholera (Petsaris et al., 2010). *V. cholerae* O1 strains are grouped into two biotypes; classical and El tor. The El Tor and classical biotypes of *V. cholerae* O1 are characterized on the basis of biochemical properties and phage sensitivity, whereas the serogroups differentiation is based on the O-antigen structure (Dziejman et al., 2002). Biotype classification is based on a wide variety of sets of phenotypic traits which include susceptibility to polymixin B, hemagglutination of chicken erythrocytes, hemolysis of sheep erythrocytes, the Voges-Proskauer test (for measuring the production of acetylmethylcarbinol) and susceptibility to phages (Udden et al., 2008). The ability of *V. cholerae* O1 and O139 to cause infection is due to the expression of two major virulence determinants; the toxin coregulated pilus (TCP) responsible for colonization of the bacteria in the host gastrointestinal environment, and the cholera toxin (CT), an exotoxin that facilitate the secretion of severe diarrhoea. The TCP and CT are located on two separate genetic elements termed '*Vibrio* Pathogenicity Island' (VPI) and the 'filamentous bacteriophage, CTXΦ phage', respectively (Shakhnovich et al., 2007). The two virulence determinants occupy two regions within *V. cholerae* chromosome in which genes encoding virulence factors are clustered. Therefore the ability of *V. cholerae* to cause infection will depend on the synergistic action of the Pathogenicity Island coupled with CTX phage (Faruque et al., 1998). Other "O" serogroups of *V. cholerae* that do not agglutinate the O1 or O139 antiserum are referred to as non-agglutinating (NAG) or non-O1/non-O139 serogroups (Eddabra et al., 2011). These strains are recognized as causative agents of sporadic and localized cases of diarrhoea, isolated from cases of infections including gastroentereritis, septicaemia, wound infection, meningitis and cholecystitis (Singh et al., 2001; Feghali & Adib, 2011). Cases of ear, wound and blood infections due to *V. cholerae*

non-O1/non-O139 have also been reported in recreational activities (Kirschner et al., 2008). The majority of *V. cholerae* O1 and O139 strains and *V. cholerae* non-O1 and non-O139 isolated in the aquatic environments are implicated to be non-pathogenic, due to their inability to express CT and TCP virulence determinants (Faruque et al., 1998; Singh et al., 2001; Dziejman et al., 2002). *V. cholerae* non-O1 and non-O139 are the most commonly isolated stains in marine environments. Some of these strains carry the CTX and TCP genes but most of the strains found in the environment lack these genes (Dzeijman et al., 2002).

2.7 Virulence genes associated with *V. cholerae* infection

The genes encoding the CT are part of the prophage (CTXΦ), while the genes that encode TCP are part of the *Vibrio* pathogenicity island (VPI) (Faruque & Mekalanos, 2003; Safa et al., 2006). TCP is a bacterial receptor for CTXΦ during phage infection and it is encoded by a gene cluster called accessory colonization factor (ACF), which is located near the virulence gene regulator ToxT (Karaolis et al., 1998; Bhattacharya et al., 2006). The gene cluster contains 15 ORFs with the major TCP subunit, tcpA (encoded by *tcpA* gene), which is responsible for colonization while other genes are needed for the formation and functioning of pillus assembly (Faruque et al., 1998). Both the TCP and CT are co-ordinately regulated by the same virulence regulatory gene called the *ToxR* (Waldor & Mekalanos, 1996).

The CTXΦ phage is a filamentous, lysogenic bacteriophage whose DNA that is generally found integrated at either one (El Tor) or two (classical) loci within the *V. cholerae* genome (Davis et al., 2001). The *ctxAB* genes encoding the A and B subunits of CT reside on the accessory element called 'CTX genetic element' with an approximate size of 7 to 9.7 kb DNA segment present on the chromosome of toxigenic *V. cholerae* (Waldor & Mekalanos, 1996). These genes (*ctxAB*) were indicated not to be the integral component of *V. cholerae* genome, but rather are elements of the genome of CTXΦ, that specifically infects *V. cholerae* (Davis et al., 2001). The genome of CTX phage contains a 4.5 kb central core region with five identified genes, which include *ctxAB*, *zot*, *ace*, *orfU* and *cep*. Located upstream of the central core is a repetitive sequence of 2.4 kb designated RS2 (Bakhshi et al., 2008). In addition the RS2 region comprised of two intergenic regions, *ig*-1 and *ig*-2, as well as the genes *rstR*, *rstA* and *rstB*, which are involved in regulation, replication and integration of the CTX phage respectively. The cumulative size of the core region and RS2 is 6.9 kb (Bhattacharya et al., 2005). The CTX phage DNA can also be flanked by one or

more copies of 2.7 kb DNA segment, RS1 region, which is very similar to RS2 but with an additional gene *rstC*. The *rstC* gene functions as an antirepressor to counteract the activity of the phage repressor protein RstR (encoded by *rstR* gene) (Bhattacharya et al., 2006; Bakhshi et al., 2008; Mantri et al., 2010). The *rstR* gene is of importance in classifying the CTX phage on the basis of sequence variation at the *rstR* and named according to their corresponding infective biotypes; CTX classical ($CTX^{class}\Phi$), CTX El Tor ($CTX^{ET}\Phi$) and CTX Calcutta ($CTX^{calc}\Phi$), for *V. cholerae* O1 classical, *V. cholerae* O1 El Tor and *V. cholerae* O139 respectively (Bhattacharya et al., 2005).

Majority of *V. cholerae* strains commonly found in the environment lack genes for CT, zonula occludence and accessory cholera toxin as well as TCP (Singh et al., 2001). The ability of nontoxigenic *V. cholerae* progenitors to acquire the CTX element is due to the presence of 18-bp *attRS*1, which allows the site-specific recombination system of the CTX element for attachment into *attRS*1. The horizontal transfer process is facilitated by TCP acting as a receptor, for entry of the CTX phage into nontoxigenic progenitors (Faruque et al., 1998). Nontoxigenic strains of *V. cholerae* carrying TCP genes are seldom detected from the environment but are susceptible to CTX phage infection, as a result when this strains enters the gastrointestinal environment, it confers favourable condition for TCP expression leading to CTX phage infection and emergence of toxigenic progenitors. Following the dispersion of these new toxigenic prototypes, under favourable conditions for vegetation, an epidemic form erupts (Igbinosa & Okoh, 2009).

A systematic study by Choi et al. (2010) have shown that in the absence of TCP, *V. cholerae* can acquire CTX phage through generalized transduction, due to the spatial dispersion of vibriophages in aquatic environments. The donor strains (*V. cholerae*) can transfer the CTX phage to acceptor molecules (i.e. *V. cholerae* O1 to non-O1/non-O139) in aquatic environment. Strains of non-O1, non-O139 possesses the ability to cause infection in the absence of the *ctx* gene, as was indicated with fluid accumulation in rabbit ileal loops (Singh et al., 2001). This was indicated by the ability of these strains to produce several other extracellular products such as NAG-specific heat-stable toxin, a thermostable direct hemolysis, Shiga-like toxin and hemaglutinin, which play a role in the disease process. Non-toxigenic *V. cholerae* non-O1 strains are more frequently isolated from the environment than the O1 strains (Grimes et al., 2009). The emergence of *V. cholerae* O139 is

assumed to have been evolved from the O1 strain to non-O1 through horizontal gene transfer of novel O-antigen genes (Singh et al., 2001; Raychoudhuri et al., 2010).

2.8 Detection methods for *V. cholerae* species

2.8.1 Classical microbiological detection assay

Routine laboratory practice for the isolation and detection of *V. cholerae* include the enrichment of a sample with alkaline peptone water (APW) for 6 to 8 hours followed by sub-culturing of the broth onto thiosulfate citrate bile salts sucrose (TCBS) agar (Lesmana et al., 1997). Some form of concentration, such as membrane filtration, is normally used prior to enrichment, since *V. cholerae* cells usually occur in relatively low numbers in the environment (Du preez et al., 2002). Following early stages of outbreaks (during outbreaks), traditional tests such as conventional culture involving the peptone growth test procedure, biochemical identification and serotyping are normally carried out to confirm the production of cholera toxin (DWAF, 2002). Confirmation of presumptive isolates on TCBS agar include serological assessment to identify the *V. cholerae* O1 serotype antigens. Serological confirmation involves the reaction of microorganism (antigen) with its corresponding antibody (Difco™ *Vibrio cholerae* antisera, 2003). Other tests that can provide important public health information include hemolysis, biotyping, molecular subtyping and antimicrobial sensitivity assays (Centres for disease control and prevention, Chapter 6).

Although culture methods is considered the gold standard, several days are needed to obtain results and the method has a very low reported sensitivity in environmental studies (0.01-1%) (Ashbolt, 2003; Lijima et al., 2004; Kim et al., 2011). Alam et al. (2007) have indicated that toxigenic *V. cholerae* can be isolated from 1% of the water samples tested during epidemic periods, with rare cases detected between epidemics. These can also be reasoned by the influence of competitive non-*Vibrio* species and non-cholera *Vibrio* species that overgrow and limit the growth of low numbers of toxigenic *V. cholerae* on the isolation media (Fykse et al., 2012). Furthermore a proportion of VBNC *V. cholerae* cells influence the limit of detection of culture method in aquatic niches (Alam et al., 2007). The number of viable cells could be further underrepresented by sub-lethally damaged organisms and fastidious uncultivable bacteria that are unable to divide and form colony forming units (Keer & Birch, 2003). As a result, the concentration may be too low for culturable detection but still high enough for the bacteria to cause infection (Girones et al., 2010).

The specificity in culture method can also be lowered by the selectivity of presumptive colonies in an agar media due to the presence of atypical colonies (Bauer & Rørvik, 2007). Lastly, culture techniques can only detect bacteria capable of dividing and forming colonies in laboratory culture media, and this is a problem since most bacteria in oligotrophic environments are unable to divide subject to unfavourable conditions (Lleo et al., 2005). Due to this limitations, the advent of molecular methods have been thoroughly investigated and formulated as a form of alternative to traditional culture approach, which are gaining much interests and recognition in accredited institutions.

2.8.2 Molecular detection methods

Molecular methods have been developed to increase the rapidity of analysis, which are able to achieve a high degree of sensitivity and specificity without the need for a complex cultivation and additional confirmation steps (Rompré et al., 2002). As an alternative to colony formation, they determine microbial viability based on demonstration of cellular integrity or activity and they include flow cytometry (FC) and fluorescent staining techniques, the exploitation of physiological responsiveness or metabolic activity and nucleic acid based analysis (Keer & Birch, 2003). Although FC offer several advantages which include multi-parameter data acquisition and multivariate data analysis, high-speed analysis and the ability to effect cell sorting by conveniently distinguishing cell types in mixed populations, it carries two main limitations; 1) it is very expensive (which can be higher than typical advanced laboratory instruments) and 2) skilled operators are required to obtain optimum or even acceptable performance (Davey & Kell, 1996). Fluorescence staining assays are based on antigen and antibody recognition, however antigens may be present in both viable and dead cells and cross-reaction with matrices may result in false-positive results. Furthermore the sensitivity of the antigen detection assays in bacteria is very low (Lùck & Liebscher, 2003; Liu et al., 2008).

For nucleic acid based techniques utilizing RNA as a starting material such, as Reverse transcriptase polymerase chain reaction (RT-PCR) and nucleic acid sequence-based amplification (NASBA), limitations such as rapid degradation of RNA and susceptibility to contamination by the background DNA can be easily attained (Lauri & Mariani, 2009). To present, PCR utilizing DNA as a starting material has gained popularity because the DNA 1) is a relatively stable molecule, 2) can be easily manipulated, 3) is present in all living cells and 4) contains genetic

information (Beneduce et al., 2007). However the success of PCR to obtain optimal fingerprinting patterns of microbial species relies on the efficiency of the DNA extraction procedure (Yang et al., 2008).

2.8.2.1 DNA Extraction procedures

To evaluate the efficiency DNA extraction procedures, commonly used criteria such as DNA yield, DNA shearing, reproducibility and representativeness, are usually considered (Yuan et al, 2012). Furthermore factors including 1) the time required to complete the extraction method, 2) the cost effectiveness of the method and 3) the safety of the chemical reagents used for preparation, are important for routine laboratory application (Yang et al., 2008). The complex microbial flora and the consistent variability and composition of raw wastewater will most likely result in low DNA yield (Lemarchand et al., 2005). This is due to relatively high concentration of interfering substances such as humic acids, metal ions, bile salt and cholerythrin which can subsequently inhibit *Taq* polymerase during PCR (Picard et al., 1992; Tebbe & Vahjen, 1993; Tsai et al., 1993; Dionisi et al., 2003;). Raw sewage samples frequently contain relatively high concentration of humic acids which interfere with PCR by lowering its sensitivity, leading to false negative results (Picard et al., 1992; Tsai et al., 1993; Cilliers et al., 2000; Alm et al., 2000; Dionisi et al., 2003).

The most commonly used approach to remove contaminants from environmental samples involves dilution of samples to reduce the impact of PCR inhibitors. However Tsai et al. (1993) have indicated that dilution result in low DNA yield, leading to reduction in PCR sensitivity. For the removal of humic acid, Jiang et al. (2005) included bovine serum albumin as PCR facilitators to relieve inhibitors during PCR. Although these additional steps improve the DNA recovery, they add extra costs to the extraction procedure (Cilliers et al., 2000). Attempts to use columns such as Saphedex-G200 and Elutip d column have shown to be successful only in short DNA fragments (Pichard et al., 1992; Tsai et al., 1993). The inclusion of alpha-casein into the extraction procedure was also shown to be beneficial in relieving inhibitors by acting as a chelating factor against metal ions (Boom et al., 1999). But alpha-casein has a negative influence on the purified DNA, as it induces conformational changes in the DNA or DNA processing enzymes. Other studies reported the inclusion of pre-enrichment step prior to DNA extraction, as a suitable approach for relieving inhibitors (Cilliers et al., 2000). The beneficial use of pre-enrichment is that, pre-enrichment not only dilute inhibitors but increases the number of cells in the reaction mixture.

The most critical step in the DNA procedure is the lysis of bacterial cells, since some microorganisms' are resistant to classical techniques (Picard et al., 1992). Pitcher et al. (1989) have shown that the use of guanidium thiocyanate (GuSCN), which is a strong protein denaturant (including environmental nucleases), proved to be successful for the lysis of prokaryotic and eukaryotic cells. The use of homemade DNA spin column by Borodina et al., (2003) combined with Boom et al., (1990) method have made the success of simple, more efficient DNA extraction procedure. In this method cell lysis is carried out with a lysis buffer containing GnSCN, Trishydrochloride, EDTA and Triton X-100. GuSCN, as a chaotropic agent, destabilizes the protein structure either by binding directly to the native protein structure or modifying the solvent-binding properties (Abdulova et al., 2002; Salvi et al., 2005). EDTA is included in the lysis buffer to chelate magnesium ions and destabilizes the cell wall structure and is also a cofactor for nucleases (Zidani et al., 2005). Triton X-100 is a non-ionic detergent used in biochemical applications to solubilise proteins (Sigma). Celite in a diatom suspension facilitates further purification of the DNA by forming celite-DNA complex, thus removing mainly polysaccharide contaminants (Abdulova et al., 2002). Washing of celite-DNA complex with washing buffer followed by DNA precipitation with 70% ethanol, the DNA can then be dissolved and eluted with an AE buffer (Abdulova et al., 2002). Treatment with acetone aids in the removal of proteins, RNA and inhibitors such as bile salt and cholerythrin commonly found in stools samples (Yang et al., 2008). With this method, the DNA of high purity, high molecular mass and double strand can be attained and used as a template for PCR.

2.8.2.2 Polymerase chain reaction

Polymerase chain reaction remains the most used molecular technique due to its versatility, high specificity and sensitivity, and low costs with a selectivity of amplifying a DNA fragment that has a sequence shared only by particular species or strains (Beneduce et al., 2007). Microbial species are detected by amplification of gene sequences unique to that organism with a theoretical sensitivity that, only a single intact nucleic acid template is needed to amplify a target sequence sufficiently to be visualized by electrophoresis gel (Cilliers et al., 2000). Its advantage over microbiological techniques is that it overcomes the drawbacks which include cultivation, isolation of presumptive colonies and serotyping, which will require several days to obtain results and high level of analyst competence. Furthermore, confirmation of culture method results can be quite

expensive when processing a large number of samples (Nandi et al., 2000). Instead PCR eliminate colony confirmation steps by potentially detecting bacterial targets directly from enrichment broths (Bauer & Rørvik, 2007). Therefore PCR stand-out as the best alternative method of detection due to its ability to detect pathogens in water below the detection limit presented by conventional detection methods (culture) (Girones et al., 2010). Various determinants including strains, serogroups, biotypes and virulence factors detected directly in enrichment broths, in a single multiplex reaction, have been reported in the literature (Espiñeira et al., 2010).

The success of the PCR detection assay is facilitated by proper designing of primers specific for a certain portion of the gene sequence. Several studies have reported specific primers for the detection of *V. cholerae* using gene targets specific for virulence factors, serogroup determinants as well as species specific determinants (Cilliers et al., 2000; Goel et al., 2007; Bielawska-Drózd et al., 2011). This include oligonucleotide primers specific for gene sequences such as the cholera toxin and toxin coregulated pilus genes, functional housekeeping genes specific for *V. cholerae* species, biotype specific genes and serogroup specific *V. cholerae* genes (Miyagi et al., 1999; Pourshafie et al., 2000; Pal et al., 2006; Khuntia et al., 2008; Okada et al., 2010). Example of some of the recently published *V. cholerae* gene targets for use with PCR methods are listed in Table 1.3 below.

Table 1.3 Examples of the literature published genes for *V. cholerae* PCR

Type of strain	Gene name	Function	Reference
V. cholerae species	*groEL*	Chaperonin	Fykse et al. (2012)
	hlyA	Hemolysin/cytolysin	Cinar et al. (2010)
	toxR	Central regulatory protein ToxR	Fykse et al. (2007)
	mdh	Malate dehydrogenase	O´shea et al. (2004)
	SodB	Superoxide dismutase	Ntema et al. (2010)
	Ompw	Outer membrnae protein	Nandi et al. (2000)
V. cholerae O1 and O139	*rfb*	O-antigen biosysnthesis	Hoshino et al. (1998)
V. cholerae virulence genes	*ctxA*	Cholera toxin (CT) subunit A	Espiñeira et al. (2010)
	tcpA	Toxin coregulated pilus	Fykse et al. (2007)
	zot	Zonula occludence toxin	Singh et al. (2001)
	ace	Accessory cholerae toxin	Singh et al. (2001)

Although multiple targets can be multiplexed and detected in a single reaction with PCR, it is still considered labour intensive and time consuming as the agarose gel electrophoresis step is required for confirmation of amplicon products (Kim et al., 2011). Furthermore mispriming and gel-electrophoresis-based detection decrease the sensitivity and specificity of the PCR-based monitoring (Lee et al., 2006). As a result, a more improved PCR technique (real time PCR) has been formulated which includes real time detection fluorescence chemistries, eliminate the gel electrophoresis step and improve the sensitivity and specificity of the detection assay.

2.8.1.3 Real time polymerase chain reaction

Real time PCR combines the end point PCR with fluorescence detection chemistries to detect the amplicon product accumulation in real time, with the fluorescence data collected in each PCR amplification cycle (Smith & Osborn, 2009). With this technique, amplified products are detected by fluorescence intensities generated from the dyes attached to oligonucleotide probes bound specifically to the amplified product during thermocycling (AmpliSens® *Vibrio cholerae*-FRT PCR Kit, Russia). It provides high speed, high sensitivity and potential for automation (Jyoti et al., 2010). Its sensitivity is very high and even low levels of pathogenic microbes can be directly detected in the environmental samples (Fykse et al., 2007). The real time chemistries allow product detection in PCR amplification during early phases of the reaction. With real time PCR, high resolution yield can be attained with a precise discrimination of as little as 2-fold difference between amplicon products. This is advantageous as end point PCR are based on size discrimination which may not be precise, as compared to high resolution obtained in real time chemistries (Applied Biosystems™, SA). Furthermore, traditional PCR is time-consuming and laborious during product characterization as opposed to real time PCR, which is faster and allow automation (Gubula & Proll, 2006). As a result, the risk of exposing the amplified product to the environment and subsequent contamination observed in conventional PCR is eliminated as the nucleic acid amplification and detection steps are performed simultaneously in the same vessel (Epsy et al., 2006). With real time PCR, high sensitivity can still be attained even with the detection of genes in multiplexing (Huang et al., 2009).

The real-time PCR assays developed for the detection of *V. cholerae* genes have been reported in literature (Gubula, 2006; Gubula & Proll, 2006; Blackstone et al., 2007; Huang et al., 2009). However the majority of published reports detect not more than two genes (duplex assays)

simultaneously (Huang et al., 2009), with only few studies reporting quadruplex assays (four multiplex assays) (Gubula, 2006; Huang et al., 2009). These studies have reported real time PCR assays for the detection of *V. cholerae* gene targets including species-specific, serogroup and virulence determinants with successful specificities and sensitivities.

Although PCR present numerous merits in succeeding conventional culture approach, the drawbacks of PCR is its protocol to lack standardization. This is because all the existing PCR assays are in-house optimized assays using different; 1) primer and/or probe sets, 2) reaction conditions and 3) protocols of detection. Furthermore, most of the published PCR in literature use different DNA extraction methods that are either commercialized or in-house optimized (Apfalter et al., 2001). These drawbacks render the PCR assay difficult in finding the solution of standardizing the assay. The prerequisite for a non-commercial published PCR protocols to be adopted as a standard in scientific literature require to be non-proprietary and to be validated through a multicentre collaborative trial as a form of blinded proficiency test in different accredited scientific laboratories (Hoorfar et al., 2003). The most conventionally used criteria to validate a newly developed PCR assays for use as a routine diagnostic tool; involve the inclusion of a process internal amplification control (IAC) (Rodríguez-Lázaro et al., 2004).

Process internal amplification control (IAC) is a nontarget DNA that is included in the same reaction tube containing the test sample, which can be co-amplified simultaneously with the target DNA sequence. The IAC uses its own primer and/or probe mix and do not interfere with the amplification of the target DNA, even at low copy number. Its presence in the PCR reaction monitor inhibition that can result from 1) malfunctioning of the thermal cycler, 2) poor polymerase activity, and 3) any inhibitory substance that may be present in the sample matrix (Hoorfar et al., 2003). Inhibition by sample matrix can cause PCR inhibition by preventing the amplification of the target template, resulting in false negative results and inaccurate quantification (Nordstrom et al., 2007). Some of the most successful IAC have been developed and published in literature and they include nontarget gene sequences such as rRNA and *gfp* (Murphy et al., 2007; Ntema & Barnard, 2012). Inclusion of internal controls such as *gfp* into the PCR procedure provides quality assurance and increases the reliability of the assay as it monitors the detection procedure, from DNA extraction through to amplification to the detection step (Murphy et al., 2007). The significant value of *gfp* gene as an IAC is its ability to fluorescence without exogenous cofactors

and substrates, as it contains all the information necessary for the posttranslational synthesis of the chromophore (Chalfie et al., 1994; Tsien, 1998). It is advantageous as its detection does not require either a complex media or expensive equipment, unlike other genetic markers (Errampalli et al., 1999). The application of *gfp* gene has been successful in several reported PCR assays including *V. cholerae*, *Listeria monocytogens*, *Salmonella enterica* (Murphy et al., 2007; Ntema et al., 2012).

The present study aimed to investigate the occurrence and distribution of *V. cholerae* in wastewater samples sampled from 12 selected WWTP situated in the Gauteng province. This was conducted using four PCR assays as diagnostic detection tools, in inter-laboratory collaborative trial experiments, conducted in two separate laboratories. Active monitoring setting of 'accredited' PCR assays was evaluated to confirm its reliance and applicability with the aid of previously published PCR assays. It is anticipated that the study will outline a coherent basis of 'accredited PCR assay' in active monitoring setting as well as to motivate the standardization of PCR assays.

3 The study

3.1 Rationale for the study

The occurrence and distribution of *V. cholerae* in South African wastewaters have been reported in numerous reports, with subsequent cases of cholera been recorded. As a result, commercial, ISO 17025 accredited water testing laboratories have optimized their own PCR protocols and adopted them routine diagnostic tests for routine monitoring of *V. cholerae* in wastewater samples. Although these assays have shown reproducible sensitivities and specificities, further experimental confirmations with reference to other detection methods are needed to confirm their efficiencies. This is to ascertain that the method used in active monitoring setting; is validated and reproducible, to produce reliable results. For this reason, collaborative, inter-laboratory proficiency testing schemes with other published PCR assays are needed; to provide coherent basis for comparison as well as to provide reliance of diagnostic detection tool, in active monitoring purposes.

3.2 Research question

The research question can be broken down into three parts and becomes:

1. Do *V. cholerae* occur in wastewater samples of selected WWTP investigated in the present study?

2. Can molecular PCR be adopted as reliable diagnostic tool for the detection of *V. cholerae* in selected WWTP; in active monitoring context?
3. Can inter-laboratory collaborative trial experiments be adopted as a means to confirm the reproducibility and reliability of PCR assays as routine diagnostic detection tools?

3.3 Aim

The aims of this study were to:

- Compare the efficiency of the four PCR assays from the two laboratories; in detecting *V. cholerae* and *Vibrio* species in wastewater samples.
- Confirm the reproducibility and reliability of PCR assay for use in active monitoring context.
- Confirm whether inter-laboratory comparison of diagnostic detection tools motivate the standardization of PCR results.

3.4 Objectives

The objectives of this study were to;

- Compare the detection efficiency of the four PCR assays from two laboratories in detecting *V. cholerae* in wastewater samples
- Compare the efficiency of two DNA extraction methods on whether they influence the efficiency of detection of the four PCR assays
- Confirm the reproducibility and reliability of the PCR assay results with reference to internal process amplification controls included in some of the PCR assays
- Confirm the applicability of the four PCR assays in active monitoring context; based on their overall consistency and efficiency of PCR results, in detecting *V. cholerae*
- Determine whether inter-laboratory collaborative trial experiments motivate the standardization of PCR assay.

CHAPTER 2

METHODOLOGY

1 Materials

1.1 Chemicals

All chemicals used were of molecular grade, were bought from commercial sources and were used without any further purification. The names of commercial sources for suppliers of all laboratory consumables, including materials and chemicals, are listed in the appendix 1A Section.

2 Methods

2.1 Bacterial strains

Bacterial strains of toxigenic *V. cholerae* O1 and O139 used as positive controls for the study were obtained from the National Health Laboratory Services (NHLS), American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC). All the strains were stored at -70°C on Microbank™ cryovials. The strains were grown on nutrient agar or in nutrient broth at 37°C and were used in every PCR experiment.

2.2 DNA extraction procedure

DNA extractions were done following a modification of the method reported by Boom et al., (1990) using the spin columns (Borodina et al., 2003) as described by Ntema et al. (2010).

2.2.1 Preparation of spin columns

The columns used for DNA extraction in this study were prepared following a protocol by Borodina et al. (2003). Briefly, silica membranes were cut out from GF/F borosilicate glass fiber paper by a 7-mm wad punch. The cap of a 0.5 ml microcentrifuge tube was cut off, leaving a small 'tail' (Figure 2.1). Two holes were punched at the bottom of the tube by red-hot needle. Two silica membranes were then tightly inserted into the bottom of the tube. The columns were autoclaved before use. For loading and washing, the column was placed in a reusable 2 ml screw-capped microcentrifuge tube. For DNA elution, the column was placed in a 1.5 ml tube.

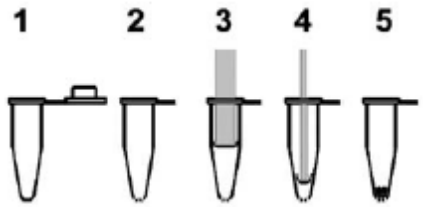

Figure 2.1 Preparation of homemade spin columns. 0.5 ml microcentrifuge tube (1) used for the experiments; cutting of caps leaving the small tail and making the holes (2); sequential steps of filter insertion (3-5) (extracted from Borodina et al. 2003).

2.2.2 Preparation of chemicals for DNA extraction method

The DNA extraction procedure used in this study follows the protocol described by Boom et al., (1999) as optimized by Ntema et al. (2010). The DNA extraction method makes use of chemicals such as lysis buffer, wash buffer and celite, and their preparation is explained as follows:

Lysis buffer: Lysis buffer was made by dissolving 120 g of GuSCN in 100 ml of 0.1 M Tris hydrochloride adjusted to pH 6.4. Subsequently, 22 ml of 0.2 M EDTA solution adjusted with NaOH to pH 8.0 and 2.6 g of Triton X-100 were added, and the solution was homogenized.

Wash buffer: For the preparation of wash buffer, 120 g of GuSCN was dissolved in 100 ml of 0.1 M Tris hydrochloride, pH 6.4.

GuSCN dissolution: Dissolution of GuSCN was facilitated by heating in a 60 to 65°C water bath with continuous shaking. Removal of contaminating DNA from buffers (lysis and wash buffer) was facilitated by addition of 5 ml celite suspension to 50 ml of each of the buffers. The suspension was mixed and left at room temperature for at least one hour after which the suspension was centrifuged at 200 rpm for 5 min. The supernatant was transferred into sterile 50 ml polypropylene tubes, wrapped in aluminium foil and stored at 4°C. Lysis buffer and wash buffer are stable for at least 6 months at room temperature in the dark.

Elution buffer: Elution buffer (AE) was bought from Qiagen and used following the instructions detailed by the manufacturer.

Celite. Preparation of celite solution was made by adding 50 ml of H_2O and 500 µl of 32% (w/v) HCl to 10 g of Celite. The suspension was divided into aliquots in small bottles which

were then closed and autoclaved at 121°C for 15 min. The glass bottles were wrapped in aluminium and stored at 4°C. The prepared celite solution can be stable for up to 6 months at room temperature.

2.2.3 DNA extraction method

The DNA extraction protocol adopted entails the method described by Boom et al., (1990) as optimized by Ntema et al. (2010). Briefly, 2 ml of APW enrichment broth were centrifuged for 2 min at 13000 rpm to pellet the cells. Following the discarding of the supernatant, the pellet was suspended with 700 µl lysis buffer and incubated at 70°C for 10 min. Additional 250 µl 100% (v/v) ethanol was added to the lysis buffer-mixture and further incubated at 56°C for 10 min. After incubation, 50 µl of celite solution was added to the mixture and incubated at room temperature for 10 min, with occasional mixing for every 2 min. Column preparations was done by putting a clean, sterile spin column into 2 ml sterile eppendorf tubes. The mixture was loaded into the prepared columns by adding 400 µl following centrifugation for 30 sec at 13000 rpm to separate the buffer from the DNA containing celite. This was repeated twice until all the lysis mixture was fully loaded into the column. The column was washed twice with 400 µl washing buffer and centrifuged for 30 sec at 13000 rpm. The last washing wash step was done with 400 µl 70% and centrifuged for 30 sec at 1300 rpm, followed by drying of the columns by centrifugation for 2 min at 13000 rpm. The columns were transferred to clean, sterile 1.5 ml eppendorf tubes. Elution of the DNA from the celite was conducted by adding 100 µl of AE buffer, followed by incubation at 65°C for 2 min. This was followed by centrifugation for 2 min at 13 000 rpm after which the columns were discarded. The AE buffer containing DNA was collected into the 1.5 ml eppendorf tube. The extracted DNA was used as a template for PCR or stored at -20°C until further use.

2.3 Polymerase chain reaction

Culture-dependent PCR methods were used to detect and identify total and pathogenic culturable *V. cholerae* in all PCR experiments. The culture-dependent PCR method was done by enriching cells in 100 ml alkaline peptone water (APW) broth for 16h at 37C and is explained in detail in Section 2.4.2 below. After incubation aerobic cells were obtained by collecting 2 ml of the APW culture from the top 3 mm from the surface and used for DNA extraction and PCR confirmation experiments. Four PCR detection methods were used for the detection and identification of *V. cholerae*. The first PCR method was quadriplex Conventional PCR for the detection and differentiation of *V. cholerae* O1, O139 and non-O1, non-O139 and

prediction of their toxigenic potential. The second PCR method used was two duplex TaqMan real time PCR's for the detection and differentiation of *V. cholerae* O1, O139 and non-O1, non-O139 and prediction of their toxigenic potential. The third PCR method was a Real-time PCR commercial kit for the detection of species-specific *V. cholerae.* The last PCR method was a real-time PCR assay coupled with High Resolution Melt curve (HRM) analysis utilizing Evagreen dye, which was specific for detection and differentiation between toxigenic and non-toxigenic *V. cholerae* strains. For every PCR reaction, positive, negative and non-template controls were included in every experiment and will be discussed in the subsequent sections below.

2.3.1 Multiplex PCR assays

The Multiplex PCR assay was used as previously described by Ntema et al., (2010). The Multiplex PCR was carried in quadriplex targeting four genes in a single reaction which included the *V. cholerae* species specific (*V.c SodB*) gene (Tarr et al., 2007), *V. cholerae* O1 and *V. cholerae* O139 *rfb* genes, and the cholera toxin gene (*ctxA*) (Lipp et al., 2003)**.** The primers used, sequences, and the predicted sizes of the amplicon products are shown in Table 2.1. Positive, negative and non-template controls were used in every experiment.

Table 2.1 Sequences of primers used in the Multiplex PCR reaction

Primers	Sequences (5'-3')	Amplicon size (bp)	References
V.c sodB	AAG ACC TCA ACT GGC GGTA GGA GTG TTA GTG ATC GCC AGA GT	248	Tarr et al. (2007)
ctxA	ACA GAG TGA GTA CTT TGA CC ATA CCA TCC ATA TAT TTG GGA	308	Lipp et al. (2003)
V. cholerae O1	GTT TCA CTG AAC AGA TGG G GGT CAT CTG TAA GTA CAA	192	Lipp et al. (2003)
V. cholerae O139	AGC CTC TTT ATT ACG GGT GG GTC AAA CCC GTA CGT AAA GG	449	Lipp et al. (2003)

PCR reactions were performed in a Biorad Mycycler^{TM} Thermal cycler in a total volume of 20 µl. Each reaction consisted of 10 µl 2x Qiagen m-PCR master mix (Master mix contains HotStartTaq DNA Polymerase, m-PCR buffer with 2 mM $MgCl_2$, and dNTP Mix); 1 µl 5x Qiagen Q-solution; 1-5 µl genomic DNA and PCR grade water. 0.25 µl of primer mix was used for the PCR reaction which consisted of a 10 uM primer stock for each primer listed in table 2.1 were added to the reaction mixture. The thermal cycling profile for the m-PCR was as

follows: The m-PCR reactions were subjected to an enzyme activation step at 95°C for 15 min, followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 60 sec and a final elongation at 72°C for 5 min. Positive, negative and non-template controls were used in every experiment.

2.3.2 Taqman real-time PCR assays

Two duplex real-time m-PCR assays were used for the detection of toxigenic and non-toxigenic *V. cholerae* O1, O139 and non-O1/non-O139 bacterial strains as described by Ntema & Barnard, (2013). The first duplex targeted the *ctxA* (cholera toxin) and *hlyA* (*V. cholerae* species) while the second duplex real-time PCR targeted O1-*rfb* (*V. cholerae* O1) and O139-*rfb* (*V. cholerae* O139). The two duplex real-time PCR assays were each multiplexed with the *gfp* assay with the latter serving to detect *E. coli*-GFP added as a positive process internal control. According to the principle of HANDS (Homo-Tag Assisted Non-Dimer System), all of the primers had a common tag sequence at their 5' ends that served to generate a universal primer binding sequence, and the tag was used as the universal primer (Huang et al., 2009). To enable simultaneous detection, each of the TaqMan probes was labelled with a different flourophore (Table 2.2).

Table 2.2 Sequences of primers and probes used in Taqman real time PCR reaction

Target and primer or probe	Sequence (5'3')	Conc (µM)	Amplicon size (bp)
ctxA-F	Tag-TCCGGAGCATAGAGCTTGGA	0.3	120
ctxA-R	Tag-TCGATGATCTTGGAGCATTCC	0.3	
ctxA-P	HEX-CCGTGGATTCATCATGCACCGCCACGG-IOWA BLACK FQ	0.05	
hlyA-F	Tag-ACTCGGTTATCGTCAGTTTGG	0.3	141
hlyA-R	Tag-CGCTTTATTGTTCGATGCGTTA	0.3	
hlyA-P	ROX-CCCCGATAATCTTGGGCAATCGCATCGGGG-IOWA BLACK FQ	0.05	
gfp-F	Tag-CCTGTCCTTTTACCAGACAACCA	0.05	77
gfp-R	Tag-GGTCTCTCTTTTCGTTGGGATCT	0.05	
gfp-P	HEX- TACCTGTCCACACAATCTGCCCTTTCG - IOWA BLACK FQ	0.05	
O1*rfb*-F	Tag-CCAGATTGTAAAGCAGGATGGA	0.3	203
O1*rfb*-R	Tag-GGTCATCTGAAGTACAAC	0.9	
O1*rfb*-P	FAM-CCCGGAGTTTGTAAGCCCACTACCGGG-IOWA BLACK FQ	0.05	
O139*rbf*-F	Tag-CATACCAACGCCCTTATCCATT	0.1	160
O139*rbf*-R	Tag-GCATGACTGGCATCCCAAAAT	0.1	
O139*rbf*-P	Cy5-CGGGTGAGAAAAGACAGCAATAACACCCG-IOWA BLACK FQ	0.05	
HANDS tag	GCAAGCCCTCACGTAGCGAA	1.2	

Real-time PCRs were performed using the Qiagen Rotor-Gene Q in a total volume of 20 µl. For both the multiplex real-time PCRs, each reaction consisted of 1X TaqMan Environmental Master mix 2.0, 1.2 µM universal primer identical to the common tag sequence of the HANDS primer (Table 2.3), 0.1 to 0.9 µM primer pairs for each amplicon, 0.05 µM of each of the five differently labelled probe (Table 2.3), and 2 µl of template DNA. The amplification procedure consisted of an enzyme activation step at 95°C for 10 min; 10 cycles of 95°C for 10 s, a 'touch down' cycle of 58°C for 20 s (with a 0.2°C decrease for each cycle up to 56.2°C), and 72°C for 15s; and 45 cycles of 95°C for 10 s, 56°C for 20 s, and 72°C for 15 s. Positive, negative and non-template controls were used in every experiment.

Fluorescence was recorded at the annealing step during the last 45 cycles. PCR products were detected by monitoring the increase in fluorescence of the reporter dye at each PCR cycle. Qiagen Rotor Gene Q software plotted the normalized fluorescence emitted from the relevant reporter dyes against the number of amplification cycles and also determines the threshold cycle (Ct) values i.e. the PCR cycle number at which fluorescence increases above a defined threshold level. For *V. cholerae* target genes (*ctxA*, *hlyA*, O1-*rfb*, and O139-*rfb*) Ct values of >40 were regarded as no PCR amplification detected and Ct values of <40 were regarded as positive PCR amplification. For the *gfp* component of the m-PCR assays, a mean Ct value of <32 was interpreted as no PCR inhibition (Calculated from the mean Ct value obtained when adding 10^4 CFU *E. coli*-GFP cells to 1 ml APW broth), a mean Ct values of >40 cycles as total inhibition of PCR and a mean Ct value of between ≥32.1 and ≤40 as loss of sensitivity.

2.3.3 Real-time PCR Commercial Kit

The Real time PCR commercial kit for the detection of *Vibrio* spp. was used as described by the manufacturer. The PCR reactions were run on an Eco™ real time PCR instrument in a total volume of 20 µl. Each reaction included 10 µl 2x precision™ MasterMix, 1 µl *Vibrio* spp. Primer/Probe mix, 1 µl internal extraction control primer/probe mix and 3 µl RNAse/DNAse free water. The volume of sample DNA added was 5 µl. The thermocycling profile for the PCR reactions constituted 50 cycles and included: enzyme activation step; 95°C for 10 min, denaturation; 95°C for 10 sec, and 60°C for 60 sec for data collection. Fluorescence accumulation for each sample was recorded at the end of each cycle. Positive, negative and non-template controls were used in every experiment.

The fluorescence was recorded at the end of the annealing step after 50 PCR amplification cycles. The PCR products were detected by monitoring the increase in fluorescence reporter

dye accumulation at the FAM and VIC channels. The Eco™ software v4.0.7.0 plotted the normalized fluorescence emitted from the relevant reporter dyes against the number of amplification cycles and determines the Ct value. For *Vibrio* spp gene the Ct values of >40 were regarded as no PCR amplification detected and Ct values of <40 were regarded as positive PCR amplification. The fluorescence of the primer and probe mix of the *Vibrio* spp gene was detected through the FAM channel. The internal control was detected in the VIC channel with an expected Ct value of 26 +/-3, which was interpreted as no PCR inhibition. Positive, negative and non-template controls were used in every experiment.

2.3.4 Evagreen High resolution melt real-time PCR assay

The Evagreen high resolution melt (HRM) real-time PCR assay was used as described by le Roux & van Blerk. (2011). The Evagreen HRM real-time PCR method was conducted at an independent accredited laboratory (IAL) in parallel to the PCR assays conducted at Water and Health Research Centre (WHRC) laboratory. The Evagreen HRM real-time PCR was conducted to compare and validate the PCR results obtained with the other three PCR methods (Section 2.3.1-3). The primers used with the Evagreen HRM real-time PCR specifically amplify the *ompW* (*V. cholerae* species-specific gene) and *ctxAB* (cholera toxin) genes (Nandi et al., 2000; Goel et al., 2005). The primer sequences with their expected amplicon sizes are shown in Table 2.3.

Table 2.3 Sequences of primers used in the Evagreen HRM real time PCR reaction (le Roux & van Blerk, 2011)

Target genes	Primer sequence (5'-3')	Amplicon size (bp)	Tm (°C)	References
ompW	CACCAAGAAGGTGACTTTATTGTG	588	81.27 ± 0.27	Nandi et al. (2000)
	GAACTTATAACCACCCGCG			
ctxAB	CCTGTCCTTTTACCAGACAACCA	564	77.68 ± 0.16	Goel et al. (2005)
	GGTCTCTCTTTTCGTTGGGATCT			

The Evagreen HRM real time PCR reaction was performed in 0.2 and/or 0.1 ml PCR tubes in a total volume of 25 µl. Amplification was performed in Rotor Gene 6000 rotary thermal cyclers (2-plex and 5-plex) with HRM capability. The PCR cycling conditions were as follows; polymerase activation step of 95°C for 10 min, followed by 45 cycles of DNA denaturation of

95°C of 30 s, annealing of 64°C for 30 s and extension at 72°C for 30 s. A final extension step was performed at 72°C for five min after cycling. For the differentiation and identification of the amplicon products formed, high resolution melt curve analysis was performed by lowering the temperature to 60°C for 5 min and subsequent increase to 90°C in increments of 0.1°C per second. Fluorescence was measured continuously and the melting temperature (Tm) peaks were extrapolated on the initial fluorescence curve (F/T) i.e. a plot of negative derivative of fluorescence over temperature versus temperature (-dF/dT versus T). Positive, negative and non-template controls were used in every experiment.

2.3.5 Confirmation of amplicon identity

Agarose gel electrophoresis was used to confirm that amplicon products obtained from TaqMan real time PCR, Evagreen HRM real time PCR and conventional PCR were of the expected length of gene targets. DNA was analysed on a horizontal agarose slab gel [2% (w/v)] with ethidium bromide (0.5µg/ml) in TAE buffer (40 mM Tris acetate; 2 mM EDTA, pH 8.3). Electrophoresis was done for 1-2 h in electric field strength of 80 V, and the DNA was visualized using ultra-violet light transluminator and the images were captured using Bio Imaging System. The relative sizes of the DNA fragments on electrophoresis gel were estimated by comparing their electrophoretic mobility with that of the standards run [100 bp makers (Fermentas®) with the samples on the gel. This procedure was followed for all the experiments except were stated differently.

The identity of suspected amplification products were also sent for sequencing and alignment of sequencing products obtained with the existing sequence database (GeneBank NCBI, BLASTn option).

2.3.6 Specificity and sensitivity of the PCR assays

The specificity and sensitivity of the Multiplex PCR, Taqman real-time PCR, and Evagreen HRM real-time PCR have been previously reported by Ntema et al. (2010), Ntema & Barnard (2013) and le Roux & van Blerk (2011), respectively. The sensitivity of the Conventional PCR in pure cultures was reported to be 60 CFU per reaction from pure culture as well as in environmental water samples. For the Taqman real-time PCR, the sensitivity was also reported to be similar in both pure cultures and environmental wastewater samples, which was indicated to be 20 CFU per reaction. The reported sensitivity of Evagreen HRM real time PCR assay was indicated to be 2 CFU per reaction in enrichment broths, of which 6 CFU per reaction could

also be attained without enrichment (i.e. direct PCR) (le Roux & van Blerk, 2011). For the real-time PCR commercial kit, the specificity of the primers and probes was reported to have a 100% homology with a broad range of clinically relevant reference sequences as indicated on comprehensive bioinformatics analysis. The specificity of the primers alone was reported to have 100% homology with all the reference sequences included in the NCBI reference database with a very broad quantification profile. The primers and probes were designed specifically for targeting the *Vibrio* spp. gene.

The specificity and sensitivity of the Taqman real-time PCR, HRM Evagreen real-time PCR and Multiplex PCR assays have been reported in literature. This study thus tested the efficiency of the four PCR assays based on comparison of the detection rate, concurrent detection rates and percentage difference of the four PCR assays in detecting *V. cholerae* (and *Vibrio* species) in the wastewater samples.

2.4 Environmental sample analysis

2.4.1 Study site and Sampling

Twelve wastewater treatment plants (WWTP) from Gauteng province were sampled. The samples were collected weekly and/or every two weeks for a period of 5 months from raw influents (upstream) to the final (treated) effluents (downstream) (July to November 2012). Wastewater samples were collected in a sterile 150 ml specimen container by the relevant laboratory personnel (from Independent Accredited Laboratory) and transported in cooler boxes containing ice bags to the two respective laboratories; Water and Health Research Centre (WHRC) and Independent Accredited laboratory (IAL), for analysis and processing. This was done to allow comparison of data generated between the two laboratories.

2.4.2 Analysis of samples

Analysis of samples was performed in a total of 136 wastewater samples (comprising of equal parts of raw and final effluents) collected from twelve selected WWTP by IAL laboratory personnel as described previously. Samples were transported to WHRC and IAL for analysis and processing as follows.

Briefly, one hundred millilitres of wastewater sample was filtered onto nitrocellulose membranes (0.45 µm pore size, 47 mm diameter) and the membrane enriched in 100 ml APW broth for 16-18 hours at 37°C without agitation. For every turbid sample, 20 ml of the sample

was directly added into 80 ml of APW broth and incubated as described previously. After enrichment, 2 ml APW enriched culture was collected from the aerobic phase (3 mm top surface) and aliquoted into 2 ml eppendorf tubes for DNA extraction. Following centrifugation of the APW broth at 13 000 rpm for 2 min, the culture supernatant was discarded and bacterial genomic DNA was extracted from the pellet cells following the method described in Section 2.2.3. Each laboratory performed in own DNA extraction method independently, using different DNA extraction procedures.

2.4.2.1 Water and Health Research Centre laboratory

From a total of 136 wastewater samples processed and analysed in IAL; a total of 68 extracted DNA samples, 28 enrichment samples and 40 water samples were shared and received at WHRC laboratory. Samples analysis was performed as described previously (Section 2.4.2) and the genomic DNA extraction was carried out with GuSCN method as described in Section 2.2.3. The extracted genomic DNA was subjected to PCR analysis described in Section 2.3.1-2.3.3.

2.4.2.2 Independent Accredited Laboratory

From a total of 136 samples received by IAL, 96 was processed and analysed as described previously in Section 2.4.2 (prior to PCR analysis) and additional 40 DNA samples were shared and received from WHRC laboratory. The IAL followed the same protocol as described previously (Section 2.4.2) except that the DNA extractions were done with InstaGene™ commercialized kit. The extracted genomic DNA was subjected to PCR analysis described in Section 2.3.4.

Samples were shared in singles in all experiments to obtain accurate data comparison between the two respective laboratories under investigation. Figure 2.2 below outline the overview of sample analysis described in the two respective laboratories.

Confirmation of positive PCR amplification in toxigenic or serogroup genes was conducted as follows. The aerobic phase of APW broth culture was streaked onto TCBS agar plates and incubated for 16 hours at 37°C. Presumptive *V. cholerae* colonies on TCBS agar were carefully selected and subcultured onto nutrient agar plates. Following incubation of 16 hours at 37°C on nutrient agar, DNA was extracted (Section 2.2.3) and positive amplicons were subsequently confirmed following the protocol described previously (Section 2.3.5.) in all the PCR reaction

showing positive PCR amplification in toxigenic genes. For quality control purposes positive, negative and non-template controls were included in every experiment.

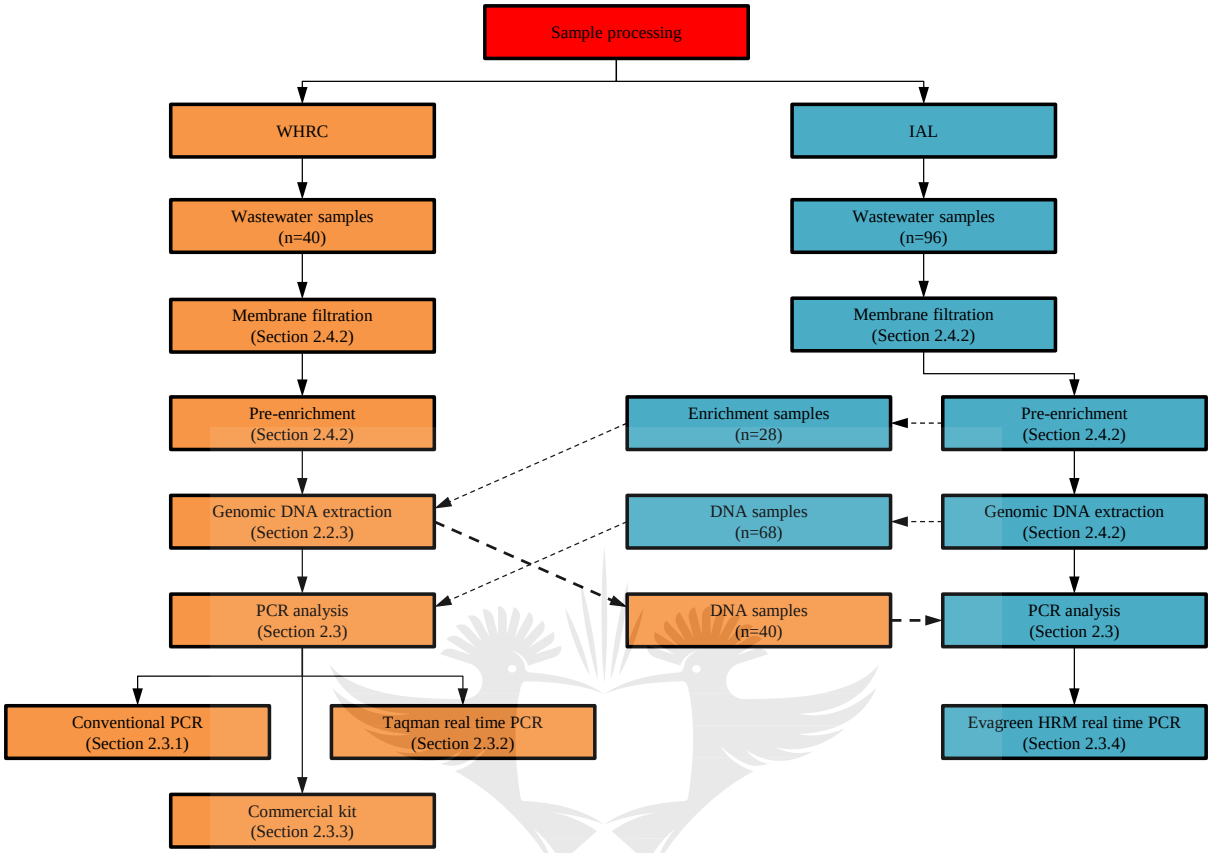

Figure 2.2. Flow diagram illustrating the experimental design for PCR analysis in environmental wastewater samples

2.4.3 Data analysis

Analysis of PCR data was performed by calculating percentages of the positive samples tested with each respective PCR assays. Percentage results was calculated by dividing the total number of positive results with the total number of samples tested multiplied by hundred, *i.e.*

$$\frac{Total\ number\ of\ positive\ samples}{Total\ number\ of\ samples\ tested} \times 100$$

All the positive PCR results were recorded following the calculations of the positive samples calculated with the equation (percentage) stated.

CHAPTER 3

RESULTS AND DISCUSSION

1 RESULTS

1.1 Introduction

The objective of this study was to compare the efficiencies of four PCR assays used for the detection of *Vibrio* species, and specifically *V. cholerae*, in wastewater samples. As outlined in Chapter 2 (Section 2.3), this collaborative, inter-laboratory proficiency testing experiment included four PCR assays used at two separate laboratory facilities; ❶ a commercial, ISO 17025 accredited water testing laboratory (IAL) and ❷ the Water and Health Research Centre (WHRC), University of Johannesburg. A total of 136 wastewater samples, comprising equal numbers of both raw influents and treated effluents, were analyzed using the different PCR assays. Each laboratory received and analyzed the samples without prior knowledge of the results obtained by their counterpart. The results were assessed in terms of the four PCR assays obtaining true positive results as confirmed by successful PCR amplification in; ❶ positive DNA template control (PTC), ❷ none template control (NTC), ❸ internal DNA extraction template control (IAC; where applicable) and ❹ process internal DNA template control (IAC; where applicable). The real-time PCR commercial kit that targets *Vibrio* species and was included as a standard method to compare the results with.

The specificity and sensitivity of the Taqman real-time PCR, HRM Evagreen real-time PCR and Multiplex PCR assays have been reported in literature (Section 2.3; Chapter 2) and were not included in the study again. This study thus compared the detection rate, concurrent detection rates and percentage difference of the four PCR assays in detecting *V. cholerae* (and *Vibrio* species) in the wastewater samples.

2 Samples received and analysis

Table 3.1 below outlines an overview of how the two laboratories (WHRC and IAL) received and processed the samples. The table indicates the total number of samples received by each laboratory, including the total number of samples from which the DNA was extracted with each respective

DNA extraction method and the type and number of samples exchanged between the two laboratories.

Table 3.1 Overview of samples analysed by WHRC and IAL laboratories

Laboratory	DNA Extraction[1]	Total samples[2]	Samples analyzed			Samples shared		
			Water	Pre-enrichment[3]	DNA	Water	Pre-enrichment[3]	DNA
IAL	InstaGene™ Matrix	136	96		40	40	28	68
WHRC	GuSCN	136	40	28	68			40

[1]DNA extraction method; [2]Total number of samples analysed; [3]APW pre-enrichment

As outlined in Table 3.1 a total of 136 wastewater samples were received and analysed by each laboratory as outlined below.

2.1 WHRC laboratory

At the WHRC, a total of 136 wastewater samples were processed and analysed with three PCR assays (as stated in Section 2.3.1-2.3.3, Chapter 2). A total of 68 DNA samples, that were enriched and DNA extracted at the IAL laboratory (with InstaGene Matrix), were received by the WHRC laboratory and PCRs were performed as stated. Twenty eight samples enriched in APW at the IAL laboratory were received by the WHRC, followed by DNA extraction (with GUSCN) and PCR analysed as stated. Lastly a total of 40 water samples were received by the WHRC and subjected to APW enrichment, DNA extraction (GUSCN) and PCR analysis as stated. The extracted DNA from these samples (40) was shared with IAL laboratory for PCR analysis to generate comparison of data.

2.2 IAL laboratory

At the IAL, a total of 136 wastewater samples were processed and analysed with PCR (as stated in Section 2.3.4, Chapter 2). A total of 96 wastewater samples were received and subjected to APW enrichment and DNA extraction with subsequent PCR analysis as stated. From the 96 samples, 68 DNA extracts and 28 APW enrichments were shared with the WHRC for DNA extraction (APW samples) and PCR analysis. Forty DNA extracts were received from WHRC as described previously.

3 PCR protocols

The expected PCR results of each of the four PCR assays when performing analysis is briefly described below. As stated previously, the sensitivities and specificities of the four PCR assays have already been reported in previous studies and were accepted as a true reflection of the PCR capabilities. These characteristics is briefly summarised below as well.

3.1 Multiplex PCR

Figure 3.1 shows the two gel pictures of the Multiplex PCR of *V. cholerae* and *V. parahaemolyticus* as shown in Ntema et al. (2010) publication. The Multiplex PCR in the present study only considered the genes specified for *V. cholerae* and multiplex in one gel in a manner that only four genes were targeted. The Multiplex PCR reported by Ntema et al. (2010) targets four genes in a single reaction which included the *V. cholerae* species specific (*V.c SodB*) gene (Tarr et al., 2007), *V. cholerae* O1 and *V. cholerae* O139 *rfb* genes and the cholera toxin gene (*ctxA*) (Lipp et al., 2003) as shown in Figure 3.1. The *sodB* gene has been tested with a variety of both cholera and non-cholera *Vibrio* strains and proved to be potent and serve as a marker for the specific-specific identification of *V. cholerae* strains (Tarr et al., 2007). The *rfb* genes for the two serogroups, O1 and O139 have previously been shown to intensively conserve the two *V. cholerae* serogroups (Stroeher et al., 1995; Stroeher et al., 1997). The *ctxA* gene sequence is diverse in that it is prevalent not only in *V. cholerae* species, but also in non-O1/O139 strains (Jiang et al., 2005).

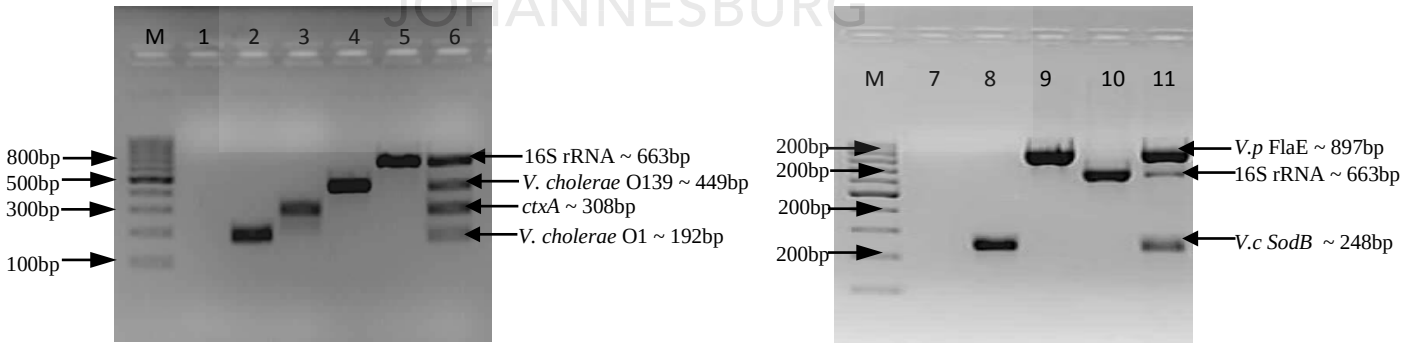

Figure 3.1 Agarose gel for the PCR products obtained for the *V. cholerae*-O1 specific gene (lane 2), cholera toxin (Lane 3), *V. cholerae*-O139 specific gene (Lane 4), the *sodB* gene (lane 8). The 100bp DNA ladder is shown in lane M and the no-DNA control in lane 1.

The specificity of the PCR reported by Ntema et al. (2010) showed that there was no non-specific amplification when tested against non-cholera *Vibrio* species strains that include; *V. parahaemolyticus, V. mimicus, V. fluvialis, V. furnissii* and non-*Vibrio cholerae* genus organisms of *Aeromonas*, *Salmonella, Bacillus, Enterococcus, Morganella, Escherichia coli*, *Shigella* and *Bacillus*. The authors also showed that the sensitivity of the PCR, combined with the APW enrichment step, can detect 60 cfu/ml of the *V. cholerae* cells per reaction.

3.2 Taqman real-time PCR

The Taqman real time PCR is made up of two triplex PCRs that target the *V. cholerae* species-specific *hlyA* gene, cholera toxin *ctxA* gene and two *V. cholerae* O1/O139*rfb* serogroup genes. The PCR was run in two triplex assays with each including the *gfp* gene, which was added as an internal process control (see Chapter 2, Section 2.3.2). The *hlyA* gene was chosen based on the reports that it is highly conserved in all classical, El Tor and non-O1 strains of *V. cholerae* (Safa et al., 2006). The specificity of the *hlyA* gene was tested for *V. cholerae* species-specific potential against non-cholera *Vibrio* species strains that include; *V. parahaemolyticus, V. mimicus, V. fluvialis, V. furnissii* and non-*Vibrio cholerae* genus organisms of *Aeromonas, Salmonella*, *Bacillus*, *Enterococcus*, *Morganella*, *Escherichia coli*, *Shigella* and *Bacillus* (Ntema & Barnard, 2013). The overall sensitivity of the PCR assay, combined with enrichment step, was shown to be 20 cfu/ml in both triplex assays.

Figure 3.2 shows the linear (correlation) relationship between the Ct values of the relative standard curves constructed with a series of 10-fold serial dilutions of heat-lysed bacterial cultures. Although Figure 3.2 shows the standard curves for quantification purposes, the present study conducted qualitative (presence/absence) experiments. This decision was based on that the study was aimed at monitoring the presence of *V. cholerae* cells in 12 wastewater treatment facilities under investigation.

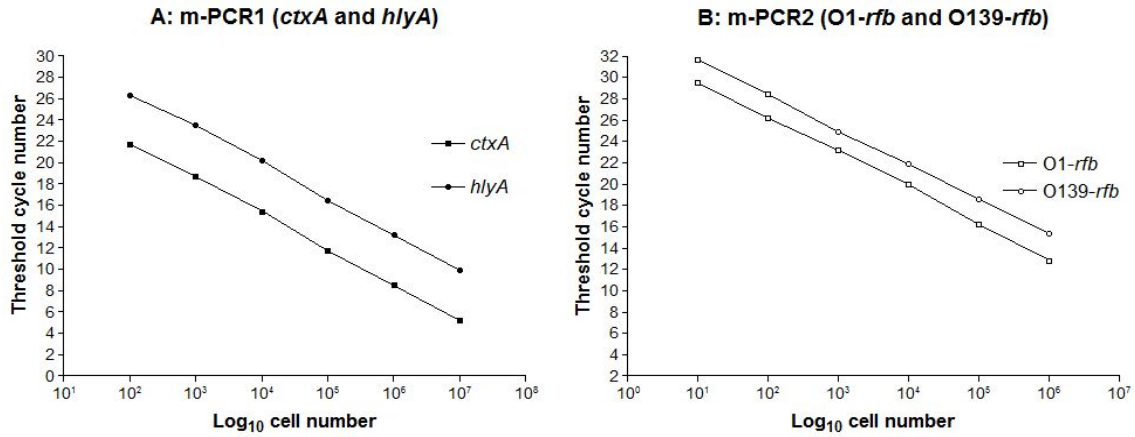

Figure 3.2 Example of standard curves of the two triplex real-time PCR reactions illustrating the linear relationship between the $\log_{10}$ values derived from ten-fold serial dilutions of bacterial cell numbers versus cycle threshold values. **A** (*ctxA* and *hlyA*), **B** (O1-*rfb* and O139-*rfb*). ●, *ctxA*, y = -3.352, r^2 = 0.997; ■, *hlyA*, y = -3.335, r^2 = 0.996; □, O1-*rfb*, y = -3.402, r^2 = 0.998; ○, O139-*rfb*, y = -3.333, r^2 = 0.993.

3.3 Real-time PCR with HRM

The Evagreen HRM real-time PCR was used as described by le Roux & van Blerk (2011) and target the *ompW* gene which is conserved in a wide spectrum of *V. cholerae* strains. The second gene targeted is the *ctxAB* gene as it has been proven to be potent (amongst other toxin produced by *V. cholerae*) and serve as a marker of cholera epidemics (Goel et al., 2005; Goel et al., 2007). As reported in the le Roux & van Blerk (2011) publication the specificity of the *ompW* and *ctxAB* primers were not tested but used as validated in the studies published by Nandi et al. (2000) and Goel et al. (2005). The size of the two genes (*ompW* and *ctxAB*) closely matched (588bp and 564bp) but well separated peaks were able to be generated when using melt analysis. Both the *ompW* and *ctxAB* gene targets generated a mean melting peaks temperatures of 80.367°C and 77.339°C for each amplicon respectively. The sensitivity of the PCR assay, combined with APW enrichment step, provided a repeatable detection of 2 CFU's after enrichment.

Figure 3.3 indicates the mean melting peak temperatures of the two target genes (*ompW* and *ctxAB*) tested in HRM real-time PCR context. The figure indicates the sizes of the two amplicons and their expected mean melting peak temperatures.

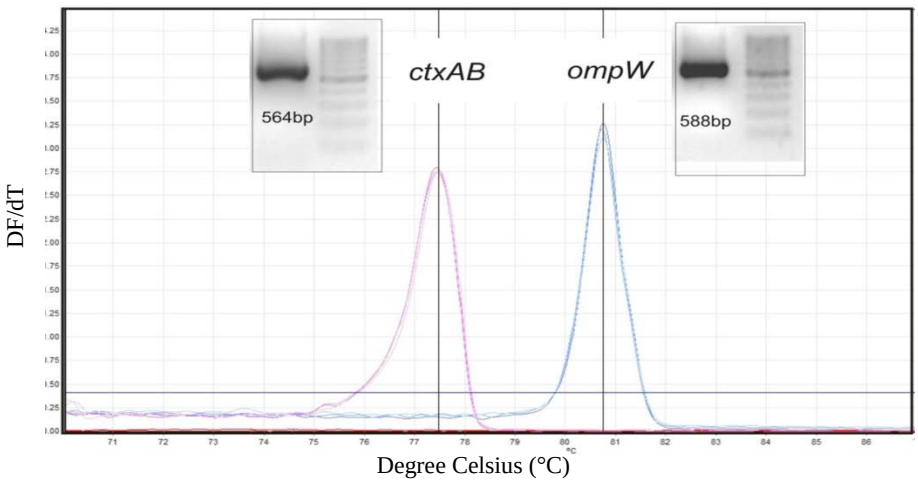

Figure 3.3 The HRM results for the *ctxA* and *ompW* amplicons depicted as dF/dT (change in fluorescence over change in time) versus temperature graph.

3.4 Real-time PCR commercial kit

For the real-time PCR commercial kit, high priming efficiencies of >95% was reported (by the manufacturer) under optimal PCR conditions with an estimated detection sensitivity of less than 100 copies of the target template (Chapter 2 Section 2.3.4). The primer and probe sequences contained in the kit is assumed to confer 100% homology in a broader range of clinically recorded sequences, confirmed by comprehensive bioinformatics database (PrimerDesign™ genesig Kit). As specified, the manufacturer only recommends that any DNA extraction protocol that is can produce an amplifiable (DNA) PCR product is deemed suitable for the kit. For this assays, qualitative (presence/absence) studies were also conducted since the study was based on testing the presence of *V. cholerae* not quantifying the amount of cells in wastewater samples.

4.1 Results of the PCR assays

With the PCR protocols presented it can be seen that the PCR compositions and target genes differ quite a bit. A summary of the PCR target genes is given in Table 3.2 below to guide the reader when the results are discussed and the methods are compared.

To validate the PCR assays results, successful PCR amplification of controls were assured prior to PCR analysis. True positive results of the four PCR assays for this study were assessed following

successful amplification of the four controls, namely; ❶ PTC, ❷ NTC, ❸ IAC-*gfp* gene (Taqman real-time PCR) and ❹ IAC-internal DNA extraction template control (real-time PCR commercial kit).

Table 3.2 Comparison of enrichment, DNA extraction and PCR protocols used for this study

Method component	Multiplex PCR	Taqman PCR[1]	HRM PCR[2]	Commercial kit[3]
Enrichment step recommended	Yes - APW	Yes - APW	Yes - APW	Not stated
Reported DNA extraction method	GuSCN	GuSCN	InstaGene kit	Not stated
Extraction control	None	*gfp* gene	None	None
Internal PCR control	None	*gfp* gene	None	DNA control
Vibrio species genes	*sodB*	*hlyA*	*ompW*	Not stated
Vibrio toxin gene	*ctxA*	*ctxA*	*ctxAB*	None
Vibrio O1/O139 gene	O1/O139*rfb*	O1/O139*rfb*	None	None

[1]Taqman real-time PCR; [2]Evagreen HRM real-time PCR; [3]Real-time PCR commercial kit

None of the samples were predicted to contain PCR inhibitors as indicated by successful PCR amplification of IAC included in both Taqman real-time PCR and the real-time PCR commercial kit. Based on these results it was assumed that none of the other PCRs would be inhibited and samples without PCR product was accepted as negative for the genes tested for. Furthermore, the presumption of true PCR results was assured by successful amplification of the PTC and no PCR products in the NTC included in each of the four PCR assays.

The results of this study showed that none of the raw influent or treated effluent samples tested positive for toxicity (*ctxA*/*ctxAB* genes) or the specific *V. cholerae* serogroups O1/O139*rfb* gene irrespective of the PCR assay used. But variability in the positive detection of *V. cholerae* (*hlyA*, *sodB* and *ompW*) as species (including *Vibrio* spp. gene) was observed among the PCR assays and is outlined below.

4.1.1 Overall detection rates of *Vibrio cholerae* and *Vibrio* species with different PCR assays

Table 3.3 summarizes the results obtained by the four PCR assays for the detection of *V. cholerae* and *Vibrio* species in a total of 136 wastewater samples. The overall detection of *Vibrio cholerae* in wastewater samples was 55.1% and 52.9% with Taqman and HRM Evagreen real-time PCR assays respectively. Multiplex PCR showed the overall lowest detection rate with only 27.2% of

samples testing positive for *V. cholerae*. Only 34.6% of samples tested positive for *Vibrio* species as shown by the commercial real-time PCR kit.

Table 3.3 Detection of non-toxigenic *V. cholerae* and *Vibrio* species with PCR assays in wastewater samples

PCR assay	Total no. of positive samples
Taqman real-time PCR	75 (55.1%)
Evagreen HRM real-time PCR	72 (52.9%)
Multiplex PCR	37 (27.2%)
Real-time PCR commercial kit	47 (34.6%)

The real-time PCR commercial kit carried a *Vibrio* species gene and was expected to target both cholera *Vibrios* and non-cholera *Vibrios*. Therefore it was expected that for every positive PCR results obtained for *V. cholerae*, the real-time PCR commericial kit would produce a positive PCR results. However the results of this investigation found that only DNA samples (i.e. enrichment and water) that were extracted with GuSCN method fell within the presumptive observations. This may imply that the efficiency of the real-time PCR commercial kit was either influenced; positively by GuSCN method or negatively, by the Instagene™ Matrix to obtain optimal PCR results. The overall concurrence detection rate of the commercial real-time PCR kit was 54.4% and 55.9% when compared with Taqman and Evagreen HRM real-time PCR assays respectively (Table 3.6). This was observed when the DNA was extracted with the InstaGene kit. Compared to the Multiplex PCR assay the concurrent detection rate of 94.1% was observed. When the DNA was extracted with the GuSCN method, concurrent detection rate of the real-time PCR commercial kit showed 82.4% and 75% when compared with Taqman and HRM Evagreen real-time PCR assays. When compared to the Multiplex PCR, concurrent detection rate of 66.2% was observed. These results imply that GuSCN method has the tendency to improve the detection efficiency of the commercial real-time PCR kit. Although high concurrence detection rates were observed between the Multiplex PCR and real-time PCR commercial kit, low efficiency in positive detection (20.6%) was still observed between the two PCR assays.

4.1.2 Detection rate of *Vibrio cholerae* and *Vibrio* species in raw and treated effluents by the different PCR assays

Table 3.4 shows the detection rates of the four PCR assays in detecting *V. cholerae* in raw influents and treated effluents. TaqMan and Evagreen HRM real-time PCR assays detected *V. cholerae* in 83.8% and 85.3% of raw influent samples respectively. The Multiplex PCR indicated the presence of *V. cholerae* in 41.2% of raw influents of which 44.1% of *Vibrio* species tested positive with real-time PCR commercial kit. Looking at only the treated effluents, 26.5% and 20.6% of the samples tested positive for *V. cholerae* with TaqMan and HRM Evagreen real-time PCR assays respectively, with only 11.8% showing the presence of *V. cholerae* when tested with the Multiplex PCR assay. The real-time PCR commercial kit indicated the presence of *Vibrio* species in 25% of treated effluent samples.

Table 3.4 Detection of *V. cholerae* and *Vibrio* species in raw and treated wastewater samples

Sample		Taqman PCR[1]			HRM PCR[2]		Multiplex PCR			PCR kit[3]
Type	**Nr**	***hlyA***	**O1/O139 *rfb***	***ctxA***	***ompW***	***ctxAB***	***sodB***	**O1/O139 *rfb***	***ctxA***	***Vibro spp***
Raw	68	57 (83.8%)	0 (0%)	0 (0%)	58 (85.3%)	0 (0%)	28 (41.2%)	0 (0%)	0 (0%)	30 (44.1%)
Treated	68	18 (26.5%)	0 (0%)	0 (0%)	14 (20.6%)	0 (0%)	8 (11.8%)	0 (0%)	0 (0%)	17 (25%)

[1]Taqman real-time PCR; [2]Evagreen HRM real-time PCR; [3]Real-time PCR commercial kit

The efficiency of the real-time PCR commercial kit in the treated influents improved enormously in the treated influents. One possible reason could be that the commercialized kit is ought to obtain optimal efficiency in samples that are pure or have minimal PCR inhibitors as reported by the manufacturer (Genesig® Advanced Kit, UK). The raw sewage influents was reported to frequently constitute relatively high concentration of humic acid substances, which subsequently interfere with PCR amplification by lowering its sensitivity and resulting in false negative results (Tsai et al., 1993; Picard et al., 1992; Alm et al., 2000; Dionisi et al., 2003). Therefore it is anticipated in the present study that the efficiency of the PCR assay will grossly depend on the suitable DNA extraction method to eliminate PCR inhibitors. Another contributing factor could be that the primer and probe sequences of the *Vibrio* species gene target contained in the real-time commercial PCR kit was reported to confer 100 % homology in a broader range of clinically recorded sequences,

conformed by comprehensive bioinformatics database (Genesig® Advanced Kit, UK). Lastly, APW enrichment has also been implicated to dilute the impact of PCR inhibitors (Lesmana et al., 1997). High detection efficiency obtained by both Taqman and Evagreen HRM real-time PCR is not a surprise as supported by their optimized reported sensitivities and specificities. The reported sensitivity of Evagreen HRM real time PCR assay was 2 CFU of *V. cholerae* cells per reaction (le Roux & van Blerk, 2011). Furthermore the *V. cholerae* species-specific *ompW* gene was proved to be highly conserved among *V. cholerae* strains belonging to wide variety of biotypes or serotypes (Goel et al., 2005). The overall optimized sensitivity of TaqMan real-time PCR was also reported to be 20 CFU *V. cholerae* cells per reaction, in APW enriched cultures (Ntema & Barnard, 2013). These results are comparable with other studies, which obtained a detection limit of 10 CFU/ml with TaqMan primer-probe set specific for *V. cholerae hlyA* gene in synthetic seawaters (Lyon, 2001). These indicate the conserved sequence of *hlyA* in a wide variety of environmental strains of *V. cholerae* species for universal use as species-specific gene. Although high specificity was observed for *sodB* gene for potential use as species-specific marker during its optimization (Ntema et al., 2010), its comparison to *hlyA* and *ompW* could be biased as its potential was never reported in real-time PCR context. This is because real-time PCR is reported to be more sensitive than conventional PCR and gel electrophoresis (Gubula & Proll, 2006). The overall low detection efficiency of Multiplex PCR could be attributed to optimized reported sensitivity of 60 cfu/ml, which is lower than any of the reported three PCR assays tested in the present study.

4.1.3 Detection rate of PCR assays in DNA, enrichment and water

When considering the efficiency of the four PCR assays, factors such as the DNA extraction methods and enrichment protocol (employed at IAL and WHRC) needs to be considered in a context on whether they influence the results of the efficiency of the four PCR assays. The results are indicated in Table 3.5 below.

From 68 DNA samples that were extracted at IAL (InstaGene kit), the highest detection of *V. cholerae* species was 58.8% and 55.9% with Taqman and Evagreen HRM real-time PCR assays respectively. Multiplex PCR could only detect 22.1% of *V. cholerae*, with 16.2% tested positive for *Vibrio* species.

From 28 enrichment samples that were enriched at IAL and DNA extracted at WHRC (GuSCN), PCR results indicated the highest detection of *V. cholerae* species with Taqman real-time PCR (67.3%) and Evagreen HRM real-time PCR (64.3%). The detection rate of Multiplex PCR was 53.6% and the real-time commercial PCR kit had the highest detection rate of 75% in *Vibrio* species.

From 40 water samples that were enriched and DNA extracted at WHRC (GuSCN), the detection of *V. cholerae* was 40% with both Taqman and Evagreen HRM real-time PCR assays. The detection rate of the real-time PCR commercial kit and Multiplex PCR was 37.5% and 17.5% respectively.

Table 3.5 Determination of *V. cholerae* and *Vibrio* species in DNA, enrichment and water

Type of sample	Total no. of samples	Taqman PCR[1]	HRM PCR[2]	Multiplex PCR	PCR kit[3]
		hlyA	***ompW***	***sodB***	***Vibrio spp***
DNA	68	40 (58.8%)	38 (55.9%)	15 (22.1%)	11 (16.2%)
Enrichment	28	19 (67.3%)	18 (64.3%)	15 (53.6%)	21 (75%)
Water	40	16 (40%)	16 (40%)	7 (17.5%)	15 (37.5%)

[1]Taqman real-time PCR; [2]Evagreen HRM real-time PCR; [3]Real-time PCR commercial kit

Although no specific tests were reported to compare the efficiencies of the two published DNA extraction methods used, their capabilities were tested on the context of producing a high quality DNA that can be amplified by any of the four PCR assays tested in the present study. As a result the DNA extraction methods were used as reported with no further tests included for quantitative or qualitative comparison and their efficiency were reflected on the efficiency of respective PCR assays. From the results obtained in the present study, it is presumed that the GuSCN method had the tendency to improve the detection efficiency of PCR assays more importantly, that of the real-time PCR commercial kit and Multiplex PCR. The critical factor of the DNA extraction method is to produce a high quality DNA that is free from PCR inhibitors and that can be easily amplified by PCR (Yang et al., 2008). Therefore the DNA produced by the InstaGene Matrix generated low

quality DNA for the real-time PCR commercial kit as compared to the high quality DNA generated with GuSCN method. One possible reason could be that the Instagene™ Matrix relies on boiling to lyse the cells and Sharbatkhori et al. (2009) has indicated that boiling has the tendency of not to extract the DNA efficiently due to cells resistant to lysis. In comparison, guanidium thiocyanate (GuSCN) is a strong protein denaturant which has proved to be successful for the lysis of prokaryotic and eukaryotic cells (Pitcher et al., 1989). This definite difference could be the main contributor to the results obtained by the two DNA extraction method used in the present study. But it should be noted that both Taqman and Evagreen HRM real-time PCR assays provided efficient sensitivities regardless of the choice of DNA extraction method used.

4.1.4 Concurrent detection rates of PCR assays in detecting *Vibrio cholerae* in wastewater samples

Table 3.6 compare the concurrent detection rates of the three PCR assays for the positive detection of *V. cholerae* in wastewater samples. Taqman and Evagreen HRM real-time PCR assays obtained a maximum concurrent detection rate of 47.1% for the positive detection of *V. cholerae* as compared to 23.5% obtained with Multiplex PCR when combined with Taqman and Evagreen HRM real-time PCR assays respectively. These results indicate a difference of 8% and 5.8% when compared to the detection rate of Taqman and HRM Evagreen real-time PCR assays respectively. A difference of more than 29% was observed when concurrent detection rate of Multiplex PCR was compared with either of the two real-time PCR assays. A concurrent detection rate of 21.3% for the positive detection of *V. cholerae* was observed with all the three PCR assays when combined.

Table 3.6 Concurrent detection rate of different PCR assays in detecting *V. cholerae* from wastewater samples

Total (%)	PCR 1	PCR 2	PCR 3	PCR 1 and 2	PCR 1 and 3	PCR 2 and 3	PCR 1,2 and 3
	Taqman PCR[1]	HRM PCR[2]	Multiplex PCR				
136 (100)	75 (55.1)	72 (52.9)	37 (27.2)	64 (47.1)	32 (23.5)	32 (23.5)	29 (21.3)

[1]Taqman real-time PCR; [2]Evagreen HRM real-time PCR

The detection rate of Taqman (55.1%) and Evagreen HRM (52.9%) real-time PCR assays did not differ considerably with their concurrent detection rate (47.1%). These results imply that the two PCR assays are best suited for routine application in active monitoring of *V. cholerae* in wastewater treatment facilities. This is because the choice of DNA extraction method, tested in the present study, did not have a considerable influence on the efficiencies of the two real-time PCR assays. The results imply that, for active monitoring of *V. cholerae* in wastewater treatment facilities, both Taqman and Evagreen HRM real-time PCR assays can be used when the DNA is prepared either with GuSCN or InstaGene Matrix.

4.1.5 Percentage difference of the PCR assays for the positive detection of *V. cholerae* in wastewater samples

Table 3.7 indicate the results of the percentage difference in detection rates of the PCR assays for the positive detection of *V. cholerae* in DNA, enrichment and water samples. When comparing the analysis of the DNA extracts that were prepared with the Instagene™ Matrix, Taqman real-time PCR detected *V. cholerae* in 58.8% of samples compared to 55.9% of samples when using HRM Evagreen real-time PCR. This is a percentage difference of 2.9%. Compared to the Multiplex PCR assay (22.1% of samples positive for *V. cholerae*) the difference was 36.7% and 33.8% respectively. This results could be attributed to the overall low efficiency (i.e. detection rate) conferred by Multiplex PCR (Table 3.3) as supported by lower concurrent detection rates obtained when results of the PCR assay was compared to the two real-time PCR results (Table 3.6). Low percentage difference in the detection rate obtained between the two real-time PCR assays is supported by the high concurrent detection rate observed between the two PCR assays (Section 3.3).

When comparing the analysis of the enrichment samples that were DNA extracted with GuSCN, Taqman real-time PCR detected *V. cholerae* in 67.3% of samples compared to 64.3% of samples when using HRM Evagreen real-time PCR. That is a percentage difference of 3% which is similar to that obtained in the DNA extracts. With Multiplex PCR, the detection of *V. cholerae* in enrichment samples was 53.6% which is a percentage difference of 13.7% and 10.7% when compared to Taqman and HRM Evagreen real time PCR assays respectively. The percentage difference here is far better to that obtained in the DNA extracts. As mentioned previously, the results indicate the efficiency of the GuSCN method to improve the detection efficiency of the

Multiplex PCR assay. Furthermore the results also indicate the efficiency of the two real-time PCR assays irrespective of the choice of the DNA extraction protocol used.

When comparing the analysis of the water samples that were enriched and genomic DNA extracted at WHRC (with GuSCN method), both Taqman and HRM Evagreen real-time PCR assays detected *V. cholerae* in 40% of samples with no percentage difference (0%) between the two PCR assays. Compared to Multiplex PCR (17.5% of samples positive for *V. cholerae*) the percentage difference was 22.5%. The percentage difference of Multiplex PCR was a far much better than that of the DNA extracted with the Instagene™ Matrix indicating the improved efficiency of the PCR assay when the genomic DNA is extracted with GuSCN method.

Table 3.7 Percentage difference of PCR assays for the detection of *V. cholerae* in wastewater samples

Type of sample (Total)	PCR assays	Detection rate (%)	Percentage difference		
			Taqman PCR[1]	HRM PCR[2]	Multiplex PCR
DNA (68)	Taqman PCR[1]	58.8		2.9	36.7
	HRM PCR[2]	55.9	2.9		33.8
	Multiplex PCR	22.1	36.7	33.8	
Enrichment (28)	Taqman PCR[1]	67.3		3	13.7
	HRM PCR[2]	64.3	3		10.7
	Multiplex PCR	53.6	13.7	10.7	
Water (40)	Taqman PCR[1]	40		0	22.5
	HRM PCR[2]	40	0		22.5
	Multiplex PCR	17.5	22.5	22.5	

[1]Taqman real-time PCR; [2]Evagreen HRM real-time PCR

It should be noted that the choice of DNA extraction method did not have any impact on the efficiency of Taqman and HRM Evagreen real-time PCR assays. Compared to Multiplex PCR significant improvement in percentage difference was observed when compared to both Taqman and Evagreen HRM real-time PCR assays. These results were based on the DNA that was extracted with GuSCN method. The DNA extraction method is an in-house optimized Guanidinium thiocyanate (GuSCN) protocol from the modification of Boom et al. (1999) that relies on spin

column for efficient extraction to relieve PCR inhibition. Furthermore, the DNA extracted with GuSCN method is collected in elution buffer, which provide a greater capacity of collecting high quality pure DNA. This is advantageous as opposed to the DNA obtained in the supernatant of the InstaGene matrix.

High percentage difference between positive detection of PCR assays was observed in DNA that was APW enriched and DNA extracted (InstaGene kit) at IAL. This could be attributed by the efficiency of the DNA extraction method to produce a desirable, amplifiable DNA that is suitable for Multiplex PCR. The overall detection efficiency of Multiplex PCR was very low with a percentage difference ranging from 10.7 to 36.7% when compared to both Taqman and Evagreen HRM real-time PCR. But it should be noted that the lowest percentage difference (less than 3) was observed, throughout the study period, between Taqman and Evagreen HRM real-time PCR assays despite the choice of DNA extraction used. Although the two laboratories independently applied similar enrichment protocol during samples analysis, discrepancies in PCR results was observed in the Multiplex PCR in water samples. Despite this observation, the majority of the PCR assay results were not influenced by the enrichment protocol. Both the two laboratories followed a gold standard of filtration of 100 ml of water in 0.45 um nitrocellulose membrane, followed by enrichment in 100 ml APW broth incubated for 16-18 at 35 to 37°C.

5 DISCUSSION

5.1 PCR assays controls

A systematic study by Bourhy et al. (2011) has shown that multicenter comparison of diagnostic detection tools is necessary to validate and increase diagnostic efficiency. In the present study, the validity of the four PCR results was shown to be successful as reflected by successful amplification of PTC, NTC and IACs. Both IACs contained in Taqman real-time PCR and real-time PCR commercial kit were successfully amplified indicating that no PCR inhibition was encountered. The results assumed that none of the four PCRs were inhibited as samples without PCR product were accepted as negative for the genes tested for, with reference to successful amplification of both PTC and NTC. Further information on IAC is discussed, in details, in PCR sections below.

5.2 Efficiency of PCR assays in detecting species-specific genes

The blinded collaborative, inter-laboratory proficiency testing study favored both Taqman and HRM Evagreen real-time PCR assays as potential diagnostic tools to be routinely employed for the active monitoring of the occurrence and distribution of *V. cholerae* in wastewater treatment plants. Although none of the samples showed the presence of toxicity genes (*ctxA*/*ctxAB*) or the specific *V. cholerae* serogroups O1/O139*rfb* genes with any of the PCR assays, variability in positive detection of *V. cholerae* as species (*hlyA*, *ompW* and *sodB*) was observed. The highest detection of *V. cholerae* was observed with both Taqman and Evagreen HRM real-time PCR assays throughout the study period, indicating the potential use of the PCR assays as routine diagnostic tests for active monitoring evaluation. When compared to Multiplex PCR, these results could attribute that real-time PCR analysis offer more sensitive results than conventional gel electrophoresis PCR as reported in other studies (Gubula & Proll, 2006). The ability of real-time PCR chemistry to obtained optimal PCR efficiency relies on the detection of product in PCR amplification during early phases of the reaction. With real time PCR, high resolution yield can be attained with a precise discrimination of as little as 2-fold difference between amplicon products. This offer an advantage as opposed to end point PCR, which relies on size discrimination that may not be precise, as compared to high resolution obtained in real time chemistries (Applied Biosystems™, SA).

As for the real-time PCR commercial kit, not much can be said as information on how the reaction should be prepared including enrichment procedure and preferred DNA extraction method was not provided by the manufacturer. Also the target gene was universal for *Vibrio* species which could be difficult to predict a wide existing spectrum of environmental *Vibrio* species. This is because environmental strains are exposed to adversities that may induce small nucleotide polymorphism in the target gene, resulting in inhibition in PCR amplification (Guy et al., 2003; le Roux & van Blerk, 2011). Additionally, the kit only state that it should be used on pure samples or with minimal inhibitors, for any DNA extraction method. But it should be noted that the kit performed much better than any other PCR assay in treated effluents indicating high specificity outlined by the manufacturer. These results may imply that only pure samples (i.e. treated effluents) are ought to be used to obtain optimal efficiency of the PCR results.

5.3 Taqmam real-time PCR

The suitability of using Taqman real-time PCR in the present study was necessitated by its protocol to include the process internal amplification control (IAC). This study followed to the protocol explained by Murphy et al. (2007) to optimize *V. cholerae* PCR protocol as published by Ntema & Barnard (2013). Genetically modified strains of *E. coli* carrying an easily fluorescing *gfp* gene (designated as *E. coli*-GFP), was used as an internal process control. The IAC used was included in both duplex, two-step PCR assays, in the form of *E. coli*-GFP cells (Section 2.3.2; Chapter 2). The *E. coli*-GFP cells were directly incorporated into APW enrichment as whole cells prior to genomic DNA extraction, in order to be co-extracted with APW enriched cells. Inclusion of IAC was exclusively for validation purposes, to monitor the assay performance from target cell lysis, DNA extraction to amplification process (Murphy et al., 2007). But it should also be noted that the primary goal of including IAC was to monitor PCR inhibition. The present study followed the same optimized PCR conditions (with IAC included) to evaluate the validity of this protocol in environmental wastewater samples. The results obtained in the present study showed successful PCR amplification with no PCR inhibition as supported by positive amplification signal of IAC in samples with no target sequence. The IAC (*gfp* gene) produced a positive PCR amplification signal of *gfp* with expected Ct values (Section 2.3; Chapter 2). The importance of including of IAC in the PCR assay is to eliminate false-negative results as they can turn the risk of the public into a thread. This is because with false-positive results, at least clarification of the presumptive results by retesting can be performed (Hoorfar et al., 2003).

5.4 Evagreen HRM real-time PCR

As for the Evagreen HRM real time PCR assay, the overall optimized sensitivity of 2 CFU of *V. cholerae* cells per reaction could be attained, but with no exogenous IAC included (le Roux & van Blerk, 2011). During its optimization (i.e. Evagreen HRM real-time PCR), the applicability of closely related (i.e. with size) primers of *ompW* and *ctxAB* genes was tested in the HRM real time PCR context to produce two separate, melting peaks. The results of melt analysis were well separated amplicon products with mean melting peak temperatures of 80.367°C for *ompW* (588 bp) and 77,339°C for *ctxAB* (564 bp). This allowed a successful multiplexing of *ompW* and *ctxAB* genes in Evagreen HRM real time PCR assay, even with high specificity in various small nucleotide polymorphism (SNP's). The optimized melting curves were also obtained in spiked

environmental water samples, although environmental strains with variations in *ompW* genes generated SNP's (le Roux & van Blerk, 2011). Similar results were also observed in environmental wastewater samples tested in the present study (Table 3.5). Studies by Guy et al. (2003) have shown that mismatches within the sequence of different strain of the target gene, is prone to mismatch of the initial design of the primer-probe set. These authors elucidated that mismatch of the primer-probe to target sequences frequently lead to inhibition, owing to lower amplification efficiency of the PCR reaction.

Evagreen HRM real time PCR did not use probes owing to several advantages such as; the assay not been subjected to limitations of: - ❶ probe design and ❷ unspecific probe binding. However variations in HRM Melting Temperature (Tm) values within *ompW* genes were still observed in some of the environmental wastewater samples tested in the present study, probably due to high sensitivity of HRM to detect small differences in sequence length and composition. Furthermore, interpretation of melting curve data requires a competent analyst, with strong background and good understanding of melt-based PCR technologies (le Roux & van Blerk, 2011). Despite the above mentioned limitations, both Evagreen HRM and TaqMan real time PCR assays were able to provide high efficiencies in detecting *V. cholerae* in wastewater samples, owing to their insignificant difference in the level of detection rates.

5.5 Real-time PCR commercial kit

The commercial real-time PCR kit was specific for *Vibrio* species in one-step assay, with internal exogenous DNA extraction control included in the PCR reaction (as provided by the manufacturer). The internal exogenous DNA extraction control was spiked directly into either extracted DNA (provided by IAL) or lysis buffer (in both enrichment and water samples), to be co-purified with the DNA samples. All the recorded positive wastewater samples tested indicated a mean Ct value of 26 +/-3 for the internal DNA extraction control, which complied with the recommended manufacturer's specifications (Genesig® Advanced Kit, UK). The Instagene™ Matrix relies on boiling to lyse the cells and previous studies have indicated that cells are frequently resistant to lysis when boiling method is employed (Sharbatkhori et al., (2009). A study by Radstrom et al., (2003) has shown that one of the ways to overcome PCR inhibition is to increase the concentration of target DNA template. The internal DNA extraction DNA template of

the commercial real-time PCR kit was added at high concentration of approximately 2000 copies, to generate Ct mean values of 26 +/-3.

5.6 Multiplex PCR

Multiplex PCR was a conventional gel electrophoresis targeting four genes in a single reaction which included; the *V. cholerae* species specific (*V.c SodB*) gene (Tarr et al., 2007), *V. cholerae* O1 and *V. cholerae* O139 *rfb* genes, and the cholera toxin gene (*ctxA*) (Lipp et al., 2003)**.** The PCR did not include IAC in their protocol. The efficiency of the PCR assays was proved not to be efficient for active monitoring of *V. cholerae* in wastewater treatment facilities when compared to other PCR assays (Taqman and Evagreen real-time PCR). Although the PCR assay was shown to be influenced by the choice of the DNA extraction method, the efficiency was still considerably low. The ability of *sodB* gene to be used as a marker for species-specific for the environmental *V. cholerae* strains needs further testing in real-time context. This is because the gene (*sodB*) was shown to be highly conserved in variety of environmental strains of *V. cholerae*, which could serve as a potential marker for species-specific identification (Tarr et al., 2007). Therefore the overall low efficiency conferred by Multiplex PCR assay in the present study can only be attributed by the low efficiency of conventional PCR as opposed to real-time PCR (Gubula & Proll, 2006; Blackstone et al., 2007; Huang et al., 2009).

5.7 DNA extraction protocols

Both GuSCN and InstaGene Matrix were efficient to produce a desirably, amplifiable DNA suitable for amplification of the PCR assays used in the present study. But variability in efficiency of the PCR assays, as influence by the choice of DNA extraction method, to detect *V. cholerae* (and *Vibrio* species) in wastewater samples was observed. The efficiency of positive detection by both Taqman and Evagreen real-time PCR was not considerably influenced by the choice of DNA extraction method as compared to other PCR assays. The efficiency of Multiplex PCR assay was shown to be positively influenced by GuSCN method. Compared to Taqman and Evagreen HRM real-time PCR assays, a percentage difference of 10.7 and 13.7 was observed in enrichment samples (that were DNA extracted with GuSCN) as compared to 36.7 and 33.8 observed in DNA samples prepared with the InstaGene Matrix. In water samples, a percentage difference of 22.5 was observed which attribute to the efficiency of GuSCN method to improve the detection

efficiency of Multiplex PCR assay. However the same cannot be said about Taqman and Evagreen HRM real-time PCR as their percentage difference ranged from 0 to 3 in DNA prepared with either GuSCN or InstaGene Matrix.

All four the PCR experiments included a 16 h enrichment step with APW broth at 37°C (Chapter 2; Section 2.3) prior to genomic DNA extraction. The purpose of including APW enrichment was aimed at serving three purposes for this study; 1) to dilute the impact of inhibitors, 2) to increase the number of viable *V. cholerae* cells present in environmental wastewaters, and 3) to minimize the chances of obtaining false-positive PCR results (Lesmana et al., 1997; Jeršek et al., 2005). Inclusion of APW enrichment prior to genomic DNA extraction is implicated to increase the number of targeted cells, which in turn, increase the concentration of the DNA template (Jeršek et al., 2005). Furthermore, with inclusion of enrichment, presumptive viable cells can be obtained at aerobic phase (3 mm top surface) to selectively discriminate viable cells as PCR alone cannot differentiate between live and dead (le Roux & van Blerk, 2011).

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

1 General discussion and conclusion

The primary drawback of PCR methods not being considered as a 'gold standard' for testing is the lack of standardization. This is due to the fact that all the existing PCR assays are in-house optimized protocols using different DNA extraction methods, primer and/or probe sets, reaction conditions and methods of detection. Most of the reported published PCR protocols recorded in scientific literature use different DNA extraction methods that are either commercialized or in-house optimized (Apfalter et al., 2001). Hoorfar et al. (2003) has indicated that the prerequisite for a non-commercial published PCR protocol to be adopted as a standard in scientific literature requires it to be non-proprietary and to be validated through a multicenter collaborative trial as a form of blinded proficiency test in different accredited scientific laboratory. Therefore collaborative, inter-laboratory comparison in the form of blinded proficiency testing is of primary importance to motivate standardization as well as to generate reliable PCR results. This is important because commercial, ISO 17025 accredited water testing laboratory have optimized PCR assays that are used as routine diagnostic tests for active monitoring purposes. Validation tests are needed to confirm the suitability of routinely used diagnostic tests. One of the ways in which this goal could be attained is to conduct blinded inter-laboratory comparison trial tests in which each respective laboratory uses their own PCR protocol that include; 2) enrichment method and 2) DNA extraction method. Each laboratory will received and analyzed the samples without prior knowledge of the results obtained by their counterpart. This is done to allow comparison of data generated between respective laboratories.

The present study investigated the efficiency of different PCR assays from two different laboratories that employed separate and/or different DNA extraction and enrichment protocols in blinded proficiency testing to detect *V. cholerae* in wastewater samples. Although the two laboratories obtained optimal PCR results, the study highlighted the importance of the DNA extraction methods influence on the efficiency of certain PCR protocols. In this study the choice of DNA extraction did not have any impact in the efficiency of both Taqman and Evagreen HRM real-time PCR, but did have an influence on the other PCR assays tested. The GuSCN DNA

extraction method was shown to improve the efficiency of both Multiplex PCR and real-time PCR commercial kit. Guanidium thiocyanate (GuSCN) is a strong protein denaturant (including environmental nucleases) that has proved to be successful for the lysis of prokaryotic and eukaryotic cells (Pitcher et al., 1989).

Although APW pre-enrichment was not expected to influence the efficiency of PCR assays in the present study due to indifference in protocols employed in each respective laboratories, discrepancies in PCR results in water samples that were enriched and DNA extracted at WHRC was observed (Table 3.6; Chapter 3). However several conclusions may attribute to these observations. Firstly a considerably higher percentage difference (as opposed to APW enrichments) observed could be that the two real time PCRs were more sensitive than Multiplex PCR and amplified the DNA. Secondly it could be attributed that APW pre-enrichment influenced the results of the PCR assay. The latter can be ruled out as the two laboratories followed a 'gold standard' preparation of filtering 100 ml of water in 0.45 um nitrocellulose membrane, followed by enrichment in 100 ml APW broth incubated for 16-18 at 35 to 37°C. Inclusion of APW enrichment prior to genomic DNA extraction is implicated to increase the number of targeted cells, which in turn, increase the concentration of the DNA template (Jeršek et al., 2005). Furthermore pre-enrichment dilute the impact of inhibitors and reduces the chances of obtaining PCR false-positive results (Lesmana et al., 1997; Jeršek et al., 2005). Lastly culture-dependent PCR allows the detection of viable cells (*V. cholerae*) directly from enrichment broths, since viable *V. cholerae* cells can be obtained in aerobic phase and subjected to PCR (Ntema et al., 2010; le Roux & van Blerk, 2011).

The most conventionally used criteria to validate a newly developed PCR assays for use as a routine diagnostic tool involve the inclusion of a process internal amplification control (IAC) (Lázaro et al., 2004). Therefore PCR assays which include process internal controls in their protocol is needed to validate the assurance that the results obtained are true-positive, with certainty that no inhibition is encountered (Lázaro et al., 2004). Process internal amplification control (IAC) is a nontarget DNA that is included in the same reaction tube containing the test sample, which can be co-amplified simultaneously with the target DNA sequence. The IAC uses its own primer and/or probe mix and do not interfere with the amplification of the target DNA, even at low copy number. Its presence in the PCR reaction monitor inhibition that can result from

1) malfunctioning of the thermal cycler, 2) poor polymerase activity, and 3) any inhibitory substance that may be present in the sample matrix (Hoorfar et al., 2003).

In conclusion, the present study investigated the suitability of PCR protocol routinely used at commercial, ISO 17025 accredited water testing laboratory (IAL) through comparison with other three published PCR assays in blinded inter-laboratory proficiency testing study. Factors such as the DNA extraction and enrichment methods were also evaluated to influence the results of the PCR assays. Results from this study showed that Taqman and Evagreen HRM real-time PCR, combined with a pre-enrichment step, have the potential to be employed in a routine laboratory setup for the active monitoring of the occurrence and distribution of toxigenic *V. cholerae* in wastewater treatment facilities. The results also indicated the importance of an appropriate DNA extraction method to ensure assay sensitivity and limit PCR inhibition. The inclusion of an IAC for Evagreen HRM real-time PCR should be considered to ensure that no false negative results are reported. This study also addressed the need for collaborative, inter-laboratory proficiency testing schemes to validate PCR detection assays for routine use in water quality testing.

2 Concluded findings

This study has shown that:

1. Both Taqman and Evagreen real-time PCR assays were potential diagnostic tests to be used in active monitoring setting based on their inconsiderable difference in; - ❶ detection rates, ❷ concurrent detection rates and ❸ their difference in their detection rates.
2. Although the choice of DNA extraction method did not have any impact on the PCR results of the two real-time PCR assays (Taqman and Evagreen HRM), GuSCN method had an impact in PCR results of both Multiplex PCR and real-time PCR commercial kit.
3. APW pre-enrichment (used in two different laboratories) was found not to have any impact on the PCR results of both Taqman and Evagreen real-time PCR assays as shown by an inconsiderable percentage difference throughout. This was further ascertained by similar preparation technique employed in both the two laboratories.
4. Real-time PCR is more sensitive than conventional PCR as reported in other studies (Lee et al., 2006; Gubula & Proll., 2006; Fykse et al., 2007).

3 Further research

This study alludes that further research should look into:

1. Continuous validation studies of PCR assays, used in commercial, ISO 17025 accredited water testing laboratory, for the endorsement of PCR protocols as potential diagnostic test for use in water quality testing. Hoorfar et al., (2003) have indicated that the universal prerequisite for a non-commercial PCR assay to be adopted as standardized method in scientific literature is required to be non-proprietary and validated, through collaborative trial in the form of blinded proficiency test, in different accredited scientific laboratories.
2. Collaborative, inter-laboratory blinded proficiency testing studies as a means of pushing for standardization of PCR protocols when taking into account; standardizing intrinsic parameters that include; type of DNA extraction protocol, method preparation for pre-enrichment, preparation of PCR reactions prior to PCR amplification. This is because all the existing in-house optimized PCR assays use different; 1) primer and/or probe sets, 2) reaction conditions and 3) protocols of detection. Furthermore, most of the published PCR in literature use different DNA extraction methods that are either commercialized or in-house optimized (Apfalter et al., 2001). This further complicates the method standardization of PCR assays.
3. Implementation of process internal control in PCR protocols used in commercial, ISO 17025 accredited water testing laboratory to ensure the reliability of the PCR results. Rodríguez-Lázaro et al., (2004) have shown that the inclusion of process internal control in PCR assays is needed to validate the assurance that the results obtained are true-positive, with certainty that no inhibition is encountered.
4. Concurrent detection rates of PCR assays in collaborative, inter-laboratory blinded proficiency testing setting as a means of validating the specificities of PCR assays to obtain reliable PCR results. Collection of concurrent detection rates of numerous PCR assays in the same sample will ascertain method reliability in active monitoring setting.

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APPENDICES

Appendix section

Section A

A1 Laboratory consumables

The names of commercial sources for suppliers of all laboratory consumables, including materials and chemicals, are listed in Table A1 below.

Table A1 Names of material and chemicals and their sources

Material/chemical	Source
Microbank™ Cryovials	Pro-Lab Diagnostics, Ontario, Canada
Nutrient Agar	Oxoid®
Nutrient Broth	Oxoid®
Guanidinium Thiocyanide	Sigma-Aldrich, Steinheim, Germany
Tris Hydrochloride	Promega, USA
EDTA Solution	Saarchem, USA
Triton X-100	Sigma-Aldrich, Steinhein, Germany
Polypropylene Tubes	Greiner Bio-One, Germany
Celite	Supelco, USA
Taqman Environmental Master Mix	Applied Biosystems
Real-Time PCR Commercialized Kit	PrimerDesign™Ltd, genesig advanced kit, UK
PCR Tubes	Corbett Research, Australia/Qiagen, Germany
Rotor Gene 6000 Rotary Thermal Cyclers	Corbett Research, Australia/Qiagen, Germany
Polycarbonate Filter Membranes	Whatman, UK
100bp Markers	Fermentas®
Sterile 150 ml Specimen Container	Plastpro Scientific, SA
Nitrocellulose Membrane	Millipore Corporation, Bellerica

Section A2

A2.1 Preparation of Media and solutions

Preparation of media and/or solutions were prepared following the protocols outlined below (as per manufacturers instruction) with no further modifications.

A2.1.1 DEHYDRATED NUTRIENT AGAR MEDUIM

A.2.1.1.1 Introduction

A medium for the determination of the microbiological flora in brewing and fermentation which can be made selective for bacteria with cycloheximide.

Table A2 Nutrient agar medium components

Typical Formula	gm/litre
Yeast extract	4.0
Tryptone	5.0
Glucose	50.0
Potassium dihydrogen phosphate	0.55
Potassium chloride	0.425
Calcium chloride	0.125
Magnesium sulphate	0.125
Ferric chloride	0.0025
Manganese sulphate	0.0025
Bromocresol green	0.022
Agar	15.0
pH 5.5 ± 0.2	

* Adjusted as required to meet performance standards

A.2.1.1.2 Directions

Suspend 75g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. If required the pH may be adjusted to 6.5 by the addition of 1% sodium bicarbonate solution.

A.2.1.1.3 Incubation

Times can vary from 2 to 14 days. Aerobic or anaerobic incubation conditions will depend on the characteristics of the organisms.

A.2.1.1.4 Storage conditions and Shelf life

Store the dehydrated medium at 10-30°C and use before the expiry date on the label. Store the prepared medium at 2-8°C.

A.2.1.1.5 Appearance

Dehydrated medium: White or pale straw coloured, free-flowing powder
Prepared medium: Blue coloured gel

A2.1.2 DEHYDRATED NUTRIENT BROTH MEDIUM

A2.1.2.1 Introduction

A general purpose fluid medium for the cultivation of micro-organisms not exacting in their nutritional requirements. Blood, serum, sugars, etc., may be added as required for special purposes.

Table A3 Nutrient broth medium components

Typical Formula	gm/litre
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH 7.4 ± 0.2 @ 25°C	

*Adjusted as required to meet performance standards

A2.1.2.2 Directions

Add 13g to 1 litre of distilled water. Mix well and distribute into final containers. Sterilise by autoclaving at 121°C for 15 minutes.

A2.1.2.3 Description

Lab-Lemco beef extract is combined with peptone and sodium chloride to form the basic bouillon described by Loeffler and other early bacteriologists. Yeast extract is added to provide vitamins and minerals to help speed the growth of most organisms.

Nutrient Broth can be enriched with other ingredients such as carbohydrates, blood etc., for special purposes. See also Nutrient Broth No.2 CM0067.

A2.1.2.4 Storage conditions and Shelf life

Store the dehydrated medium at 10-30°C and use before the expiry date on the label.
Store the prepared medium below 25°C.

A2.1.2.5 Appearance

Dehydrated medium: Straw coloured, free-flowing powder prepared medium: Straw colored solution.

A2.1.3 ALKALINE PEPTONE WATER

A2.1.3.1 Introduction

Alkaline Peptone Water is recommended by the American Public Health Administration (APHA) for enrichment of the Vibrio species from sea foods and infectious materials, such as faeces.

Table A4 APW broth medium components

Item	g/L
Peptic digest of animal tissue	10
Sodium Citrate	10

Final pH (at 25°C) 8.4 ± 0.2

A2.1.3.2 Precautions and Disclaimer

For laboratory use only. Not for drug, household or other uses.

A2.1.3.3 Preparation Instructions

Suspend 20 grams of Alkaline Peptone Water in 1000 mls of distilled water. Boil to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.

A2.1.3.4 Storage

Store the dehydrated medium at 24°C and the prepared medium at 2-8° C.

A2.1.3.5 Appearance of reference strains on APW broth

Table A5 APW broth growth performance on reference strains

Organism	Growth
Vibrio parahaemolyticus	Luxuriant
Vibrio cholerae	Luxuriant

A2.1.4 THIOSULFATE CITRATE BILE SALTS SUCROSE (TCBS) AGAR

A2.1.4.1 Introduction

TCBS Agar is recommended for the selective isolation and cultivation of *Vibrios* causing cholera and *Vibrios* which cause food poisoning. TCBS Agar promotes rapid growth of pathogenic *Vibrios* after 24 hours incubation at 37°C. Proteose peptone and yeast extract provide nitrogenous compounds. Oxgall, a derivative of bile salts and sodium citrate inhibit gram positive bacteria. Sodium thiosulfate serves as a good source of sulfur, which in combination with ferric citrate detects the production of hydrogen sulfide. For the metabolism of *Vibrios*, sucrose is added as a fermentable carbohydrate. Bromo thymol blue and thymol blue are pH indicators.

Table A6 TCBS agar medium components

Item	g/L
Proteose Peptone	10
Yeast Extract	5
Sodium Thiosulfate	10
Sodium Citrate	10
Ox gall	8
Sucrose	20
Sodium Chloride	10
Ferric Citrate	1
Bromo Thymol Blue	0.04
Thymol Blue	0.04
Agar	15

Final pH (at 25°C) 8.6 ± 0.2

A2.1.4.2 Precautions and Disclaimer

For laboratory use only. Not for drug, household or other uses.

A2.1.4.3 Preparation Instructions

Suspend 89 grams of TCBS Agar in 1000 mls of distilled water. Boil to dissolve the medium completely.

Do Not Autoclave. Cool to 50°C and pour into sterile petri plates.

A2.1.4.4 Storage

Store the dehydrated medium at 24°C and the prepared medium at 2-8° C.

A2.1.4.5 Appearance of reference strains on TCBS agar

Table A7 TCBS agar growth performance with reference cultures

Organism	Growth	Color
Vibrio cholerae	Good - Luxuriant	Yellow
Vibrio fluvialis	Good - Luxuriant	Yellow
Vbrio parahaemolyticus	Good - Luxuriant	blue
Vibrio vulnificus	Fair - good	Green yellow

Section B Preparation of solutions for DNA extraction method

B1 GuSCN method

B1.1 Lysis buffer

Lysis buffer was made by dissolving 120 g of GuSCN in 100 ml of 0.1 M Tris hydrochloride adjusted to pH 6.4. Subsequently, 22 ml of 0.2 M EDTA solution adjusted with NaOH to pH 8.0 and 2.6 g of Triton X-100 were added, and the solution was homogenized.

B1.2 Wash buffer

For the preparation of wash buffer, 120 g of GuSCN was dissolved in 100 ml of 0.1 M Tris hydrochloride, pH 6.4.

B1.3 GuSCN dissolution

Dissolution of GuSCN was facilitated by heating in a 60 to 65°C water bath with continuous shaking. Removal of contaminating DNA from buffers (lysis and wash buffer) was facilitated by addition of 5 ml celite suspension to 50 ml of each of the buffers. The suspension was mixed and left at room temperature for at least one hour after which the suspension was centrifuged at 200 rpm for 5 min. The supernatant was transferred into sterile 50 ml polypropylene tubes, wrapped in aluminium foil and stored at 4°C. Lysis buffer and wash buffer are stable for at least 6 months at room temperature in the dark.

B1.4 Elution buffer

Elution buffer (AE) was bought from Qiagen and used following the instructions detailed by the manufacturer.

B1.5 Celite

Preparation of celite solution was made by adding 50 ml of H_2O and 500 µl of 32% (w/v) HCl to 10 g of Celite. The suspension was divided into aliquots in small bottles which were then closed and autoclaved at 121°C for 15 min. The glass bottles were wrapped in aluminium and stored at 4°C. The prepared celite solution can be stable for up to 6 months at room temperature.

B2 InstaGene Matrix method

B2.1 Introduction

InstaGene matrix allows fast and easy preparation of PCR amplifiable DNA by eliminating labor intensive phenol/chloroform extraction steps. A simple cell lysis step by boiling in the presence of the matrix is sufficient. This is possible because the matrix efficiently absorbs cell lysis products that interfere with the PCR amplification process. Procedures for generating DNA suitable for PCR from bacteria using InstaGene matrix are described below.

B2.2 Contents and Storage

This bottle contains 20 ml of 6% InstaGene matrix and a magnetic stir bar. This is sufficient for 100 DNA preparations. Upon arrival, store the matrix at 4 °C.

B2.3 Warning instructions

Avoid prolonged exposure of the matrix to UV light.

The InstaGene Matrix was prepared according to the manufacturer's instructions without further purifications or modifications.

B2.4 Protocol for the preparation of genomic DNA extraction from Bacteria

The protocol described below is for the preparation of genomic DNA or episomal DNA from bacteria.

1. Pick an isolated bacterial colony and resuspend it in 1 ml of autoclaved water in a microfuge tube.

2. Centrifuge for 1 minute at 10,000–12,000 rpm. Remove the supernatant.

3. Add 200 µl of InstaGene matrix to the pellet and incubate at 56 °C for 15–30 minutes.

NOTE: InstaGene matrix should be mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. The pipet tip used should have a large bore, such as a 1,000 µl pipet tip

4. Vortex at high speed for 10 seconds. Place the tube in a 100 °C heat block or boiling water bath for 8 minutes.

5. Vortex at high speed for 10 seconds. Spin at 10,000–12,000 rpm for 2–3 minutes.

6. Use 20 µl of the resulting supernatant per 50 µl PCR reaction. Store the remainder of the supernatant at -20 °C. Repeat step 5 when reusing the InstaGene DNA preparation.

Section C

C1 Bacterial preservation and storage

Bacterial strains of toxigenic *V. cholerae* O1 and O139 used as positive controls for the study were obtained from the National Health Laboratory Services (NHLS), American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC). All the strains were stored at -70°C on Microbank™ cryovials. The strains were grown on nutrient agar or in nutrient broth at 37°C.

C2 Quality control

Bacterial strains were subcultured onto nutrient agar plates every two weeks to obtain single colonies prior to DNA extraction. Each colony was confirmed with PCR prior to subculturing into a fresh nutrient agar plate. All experiments were run following this protocol for quality control purposes.

For strains that were suspected of contamination quality control was conducted to confirm contamination. If contamination was encountered, cultures were discarded.

Section D

D1 PCR reactions

All PCR reactions were run following the recommended published protocols and the manufactures protocols. Each PCR reaction was run concurrently with each respective control that includes; PTC, NTC, DNA extraction control, APW extraction control and IAC (where applicable).

D1.1 Multiplex PCR assays

The Multiplex PCR was carried in quadriplex targeting four genes in a single reaction which included the *V. cholerae* species specific (*V.c SodB*) gene (Tarr et al, 2007), *V. cholerae* O1 and *V. cholerae* O139 *rfb* genes, and the cholera toxin gene (*ctxA*) (Lipp et al, 2003)**.** PCR reactions were performed in a Biorad Mycycler™ Thermal cycler in a total volume of 20 µl with each reaction consisted of 10 µl 2x Qiagen m-PCR master mix (Master mix contains HotStartTaq DNA Polymerase, m-PCR buffer with 2 mM $MgCl_2$, and dNTP Mix); 1 µl 5x Qiagen Q-solution; 1-5 µl genomic DNA and PCR grade water For the full detail of PCR reaction, please refer to Chapter 2; Section 2.3.1.

DNA was analysed on a horizontal agarose slab gel [2% (w/v)] with ethidium bromide (0.5µg/ml) in TAE buffer (40 mM Tris acetate; 2 mM EDTA, pH 8.3). Electrophoresis was done for 1-2 h in electric field strength of 80 V, and the DNA was visualized using ultra-violet light transluminator and the images were captured using Bio Imaging System. The relative sizes of the DNA fragments on electrophoresis gel were estimated by comparing their electrophoretic mobility with that of the standards run [100 bp makers (Fermentas®) with the samples on the gel.

D1.2 Taqman real-time PCR assays

Two duplex real-time m-PCR assays were used for the detection of toxigenic and non-toxigenic *V. cholerae* O1, O139 and non-O1/non-O139 bacterial strains as described by Ntema and Barnard, (2011). The first duplex targeted the *ctxA* (cholera toxin) and *hlyA* (*V. cholerae* species) while the second duplex real-time PCR targeted O1-*rfb* (*V. cholerae* O1) and O139-*rfb* (*V. cholerae* O139). The two duplex real-time PCR assays were each multiplexed with the *gfp* assay with the latter serving to detect *E. coli*-GFP added as a positive process internal control. According to the principle of HANDS (Homo-Tag Assisted Non-Dimer System), all of the primers had a common tag sequence at their 5' ends that served to generate a universal primer binding sequence, and the tag was used as the universal primer (Huang et al. 2009). To enable simultaneous detection, each of the TaqMan probes was labelled with a different flourophore.

Real-time PCRs were performed using the Qiagen Rotor-Gene Q in a total volume of 20 µl. For both the multiplex real-time PCRs, each reaction consisted of 1X TaqMan Environmental Master mix 2.0, 1.2 µM universal primer identical to the common tag sequence of the HANDS primer (Table 2.3), 0.1 to 0.9 µM primer pairs for each amplicon, 0.05 µM of each of the five differently labelled probe and 2 µl of template DNA (refer to Section 2.3.2; Chapter 2).

Confirmation of amplicon identity in Taqman real-time PCR was validated on agarose gel electrophoresis on whether they were of the expected length of gene targets.

D1.3 Evagreen High resolution melt real-time PCR assay

The Evagreen high resolution melt (HRM) real-time PCR assay was used as described by Roux and van Blerk, (2011). The Evagreen HRM real-time PCR method was conducted at an independent accredited laboratory (IAL) in parallel to the PCR assays conducted at Water and Health Research Centre (WHRC) laboratory. The Evagreen HRM real-time PCR was conducted to compare and validate the PCR results obtained with the other three PCR methods (Section 2.3.1-3). The primers used with the Evagreen HRM real-time PCR specifically amplify the *ompW* (*V. cholerae* species-specific gene) and *ctxAB* (cholera toxin) genes (Nandi et al, 2000; Goel et al, 2005).

The Evagreen HRM real time PCR reaction was performed in 0.2 and/or 0.1 ml PCR tubes in a total volume of 25 µl. Amplification was performed in Rotor Gene 6000 rotary thermal cyclers (2-plex and 5-plex) with HRM capability (Chapter 2; Section 2.3.4).

Confirmation of amplicon identity in Taqman real-time PCR was validated on agarose gel electrophoresis on whether they were of the expected length of gene targets.

D1.3 Real-time PCR Commercial Kit

The Real time PCR commercial kit for the detection of *Vibrio* spp. was used as described by the manufacturer. The PCR reactions were run on an Eco™ real time PCR instrument in a total volume of 20 µl. Each reaction included 10 µl 2x precision™ MasterMix, 1 µl *Vibrio* spp. Primer/Probe mix, 1 µl internal extraction control primer/probe mix and 3 µl RNAse/DNAse free water. The volume of sample DNA added was 5 µl (Chapter 2; Section 2.3.3). The Eco™ software v4.0.7.0 plotted the normalized fluorescence emitted from the relevant reporter dyes against the number of amplification cycles and determines the Ct value.