KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI, GHANA

Viral and Bacteriological Contamination of Groundwater in Peri-Urban Communities, Case Study of Dodowa community, Ghana

By
Alimamy Kolipha Kamara, (B. Eng. Honors, Civil Engineering)

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WATER SUPPLY AND ENVIRONMENTAL SANITATION

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CERTIFICATION

I hereby declare that this thesis is my own work towards the MSc degree in Water Supply and Environmental Sanitation and that, to the best of my knowledge, it contains no material previously published by another person, nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

Alimamy Kolipha Kamara	……	……..
(PG2222014)	Signature	Date

Certified by:

Dr. S. Oduro-Kwarteng	……	……..
(Supervisor)	Signature	Date

Prof. Y. A. Tuffour	……	……..
(Head of Department)	Signature	Date

ABSTRACT

Slums and Peri-urban communities in Sub-Sahara African countries often lack access to adequate and safe drinking water supply, and proper sanitation. Groundwater, predominantly through dug wells, is what often provides their water needs. Onsite sanitation systems predominantly pit latrines and open defecation in some instances is how they get rid of their faecal waste. The main objective of this study was to assess the level of safety of groundwater from dug wells and boreholes within the study area with regards to viral and bacteriological contamination. The study investigated the presence of two enteric viruses: Rotavirus and Adenovirus, and the presence and concentration of faecal indicator bacteria – *Escherichia coli* (*E. coli*), and Total Coliform in 46 dug wells, 12 boreholes, and 4 locations on a stream in Dodowa, a peri-urban town in the Shai Osudoku District in the Greater Accra Region of Ghana. A sanitary inspection of the environment surrounding each groundwater point was undertaken using a checklist of 11 potential risk factors to groundwater contamination in order to assess their risk level to contamination from their immediate environment. Virus particles were concentrated from groundwater by a glass-wool filtration process followed by detection using Nested PCR method for Adenovirus, and RT-qPCR for Rotavirus. For bacteriological analysis, the membrane filtration with culturing on agar plate was applied. The sanitary inspection result shows that 85% of the dug wells and 50% of the boreholes were at high to very high risk of contamination. For rotavirus, there was no positive result from all 18 samples from 11 boreholes, and only one out of 34 samples from 11 dug wells was positive. In effect, 1/11 dug well as against zero boreholes was positive for rotavirus. Adenovirus result shows that 22% of samples from 11 boreholes and 29% from 11 dug wells were positive. In effect, 6 out of 11 dug wells and 3 out of 11 boreholes sampled were positive for adenovirus. For the bacterial test, 90% of the boreholes and 100% of the dug wells sampled were positive for *E. coli* which indicates faecal contamination. An assessment of the influence of environmental sanitation to bacteriological quality on groundwater indicated that it has high influence on dug wells (74%) and relatively low influence on boreholes (38%). Analysis of the bacteriological result with the contamination risk assessment of boreholes and dug wells in the study area showed very weak correlation that cannot be used to validate the contamination risk assessment method applied. The conclusions drawn are that in such communities with poor sanitation, groundwater from dug wells especially, is very unsafe for consumption without adequate disinfection. Poor wastewater management coupled with poor sanitation infrastructural development and siting seemed to be the major causes of groundwater contamination in the study area. It was seen that environmental sanitation had high influence on pathogen quality of dug wells and low influence on boreholes.

DEDICATION

To God, my country, and my family

LIST OF ABBREVIATIONS AND SYMBOLS

AGI	Acute Gastrointestinal Illness
BH	Borehole
bp	base pairs
CFU	Colony Forming Unit
C(t)	cycle time (threshold)
DW	Dug well
DNA	Deoxyribo Nucleic Acid
cDNA	complementary DNA
EVD	Ebola Virus Disease
GuSCN	guanidinium isothiocyanate
HIV	Human Immuno-deficiency Virus
L	litre
ml	milliliter
mRNA	messenger RNA
NTC	Non Template Control
PBS	Phosphate buffered Saline
PCR	Polymerase Chain Reaction
RFU	Relative fluorescence unit
RNA	Ribo Nucleic Acid
RT-PCR	Reverse Transcript PCR
RT-qPCR	Real Time quantitative PCR
TCC	Total Coliform Count
vs.	versus
WHO	World Health Organisation

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I am thankful to everyone else that I have not mentioned before that gave their support to me directly or indirectly while undertaking this work.

CHAPTER 1: INTRODUCTION

1.1 Background

Safe drinking water is a basic necessity of life that humanity has continued to strive for its sufficiency. But unsafe drinking water still takes a heavy toll on human lives particularly in developing countries. The United Nations announced in 2013 that water related diseases are responsible for 80% of all illnesses and deaths in the developing world and annually account for more than 5 million deaths worldwide of which more than 50% are microbial intestinal infections, with cholera being at the top of the list. Gleick (2002) estimated that between 34 and 76 million people, mostly children, will die between 2002 and 2020 from preventable water-related diseases in spite of efforts to achieve the millennium development goal on safe drinking water. Diarrhoea, the most predominant waterborne disease has an annual episode of 4.6 million cases and 2.2 million deaths annually (WHO, 2010). 18% of the causes of child deaths in low-income countries are as a result of diarrhoea (WHO, 2011). Ingestion of water that is contaminated with human or animal faeces poses the greatest microbial risk in developing countries of Asia and Africa where limited access to both clean drinking water and proper sanitation strangulates basic standard of living (Cabral, 2010). Statistics show that in Sub-Sahara Africa, migration is from rural to urban areas, which in most cases give rise to the upsurge of informal settlements around cities and in slum areas within cities (Watson, 2009; UN Habitat, 2015). Utility services hardly cover these settlements, either because of inability of the utility, or because the people are seen to have occupied spaces unfit for habitation, or tenanted illegally (Black, 1996). For the case of the study area, it appears that rate of upsurge of dwelling houses is higher than the

utility's capability to meet rising demand. These settlements therefore rely on groundwater from dug wells or bore holes, streams, and rivers for their water needs.

The low income status of the peri-urban poor communities in the region is evidenced by poor sanitation, with onsite systems being predominantly used. There is therefore high risk of contamination of groundwater sources with pathogens which often leads to diseases outbreak (Van Geen et al., 2011). Overwhelming records have stated that waterborne diseases are prevalent in slums and peri-urban poor settlements where sanitation is low. Consequently, people with lowest financial resources and poorest hygienic facilities are liable to be affected by diarrhoeal diseases. The Global Water Supply and Sanitation Assessment Report 2000 estimated that globally, more than twice as much people lack access to improved sanitation than access to improved water supply (WHO, 2000). Not much attention is given towards effort in enhancing the quality, reliability and sustainability of water sources particularly against viral contamination (Grönwall et al., 2010). There is significant health risk of ingestion, or coming into contact with fecal contaminated water in low income settlements where diarrhoea remains a leading cause of child death (Bain et al., 2014). Graham and Polizzotto (2013) observed that the increase in use of pit latrines and groundwater in low income countries may have high consequences on public health due to pathogen and chemical contamination of ground water. Studies on the feasibility to use groundwater as water source in Sub-Sahara Africa often focus on availability and recharge capacity. Biological tests most times focus on bacteriological contamination. Viral contamination tests are uncommon in developing countries (Rigotto et al., 2010). This could be related to the

high cost (mainly due to equipment and human resources) of its undertaking (Lambertini et al., 2008).

Groundwater is the water that percolates through the surface of the earth and fills cracks and crevices in bedrocks and saturate weathered materials. The occurrence, depth, and quantity depend largely on the hydrogeology of the area and the recharge zone (Margat & van der Gun, 2013). By nature, microbial contaminant-free water falls on the earth surface as precipitation, washes over our environment, percolates through the soil and is finally stored as groundwater. The quality of the water as it gets underground is influenced by our environment both atmospheric and land surface. This influence is determined by the geochemistry of the soil, the hydrochemistry of the transporting water, and the surface chemistry of the pathogen being transported (Foppen & Schijven, 2006) and also on the environmental sanitation. Microbial contamination by bacteria, viruses, protozoa, and helminthes can occur in poor sanitary environments. Human pathogens mostly come from infected individuals and can get into water bodies when these systems are exposed to faecal contamination (Howard et al., 2000). Many waterborne pathogens particularly viruses are not readily detectable (Grabow, 1996). Enteric viruses and bacteria are found in the intestines of human and warm blooded animals and are often transmitted through the fecal oral route. Their rate of infection is higher in sub-Saharan Africa probably due to the large scale of unimproved sanitation and hygienic conditions coupled with low quality of drinking water especially in low income settlements (Bosch & Bosch, 1998). Compared to other enteric pathogens, a viral infective dose is very low. A single rotavirus particle is capable of triggering human infection (Santamaría & Toranzos, 2003). Enteric viruses can survive for months in the water matrix and still

remain infective than most intestinal bacteria (Espinosa et al., 2008; Krauss & Griebler, 2011). Coliform bacteria are unlikely to cause infection. However, their presence (*E. coli* for instance which is widely applied) is an indication of the presence of infectious bacteria such as Enteric viruses, bacteria and protozoa that are responsible for waterborne diseases outbreaks in slums of developing countries (Katukiza et al., 2013).

An assessment of the presence of common waterborne viruses and bacteria liable to find their way into groundwater locked under settlements with unimproved sanitation will provide an insight to necessary decisions required locally to protect groundwater sources of these communities from pollution. This study targeted two enteric viruses: Rotavirus and Adenovirus, because of the health risk associated with them in urban poor communities. The study also targets *E. coli* which is an indicator of fecal contamination associated with poor fecal sludge management. The study area selected for this research is Dodowa Town located in the Greater Accra Region of Ghana. It is a peri-urban poor community with a history of waterborne disease outbreaks as recently as August 2014 (The Ghanaian Times, 28th August, 2014).

1.2 Problem statement and study justification

Ground water, like surface water, is subject to all forms of contamination despite the common perception that it is naturally free from contamination. The rate and spate of population growth in peri-urban communities is high in Sub-Sahara Africa, so too is the dependency on groundwater because of unavailability or inadequacy of pipe borne water supply from public utilities. Access to proper sanitation is often low and outbreaks of waterborne diseases are common in these communities. There is little information on

human enteric virus concentration on groundwater under settlements with poor sanitation system. The percentage of households using groundwater for cooking and drinking purposes (35% and 17% respectively) in the study area is quite substantial, and the number (31%) that do not treat the water before use (Adjei, 2015) is scaring. This statistics further provided a strong case for investigation of the microbial safety of groundwater in such communities.

1.3 Research objectives

General Objective

Because contamination of water sources with human enteric viruses is common in communities with poor sanitation systems (Graham & Polizzotto, 2013), it is expected that the ground water sources, especially shallow dug wells in such communities are contaminated with enteric waterborne viruses and bacteria. In view of this hypothesis, the general objective of this study is:

To assess the level of safety of groundwater from dug wells and boreholes in a peri-urban poor settlement in Ghana with regards to viral and bacteriological contamination.

Specific Objectives

1. To assess the sanitary conditions and risk of pathogen contamination of dug wells and boreholes in the study area.
2. To test for the presence of Rotavirus, Adenovirus and *E. coli* in the groundwater sources of the study area and to assess their spatial variability.
3. To assess the influence of environmental sanitation on the bacteriological and viral quality of groundwater in the study area.

1.4 Research significance

Sustainable groundwater management at local level can be enhanced by identifying the associated health risk in poor environmental sanitation condition. The result of virus and faecal indicator bacteria concentration will give an indication of their load variation between boreholes and dug wells in such sanitary environment. This research is part of a large project (T-group project) which seeks to unlock the potential of groundwater in poor communities in Sub-Sahara Africa, with a general aim of complementing any social, technical and political changes needed for the enhancement of a sustainable urban groundwater management system in the region. The data will also be essential for Quantitative Microbial Risk Assessment of groundwater in such community without access to improved sanitation system.

1.5 Choice of study area

The choice of study area was a settlement that extensively or partly uses groundwater as a source of water supply, and at the same time has some level of sanitation challenge. The two conditions were considered because of their interrelationship. Some data such as the usage of groundwater have been sourced from other components of the T-group's set of researches that are being undertaken alongside within the study area of Dodowa.

1.6 Key terms

Certain terms related to the research title and objectives that have been commonly used may need to be defined in the context of this research:

Contamination: presence of pathogenic microbial cells in surface or groundwater sources.

Groundwater: water below ground level.

Peri-urban community: used interchangeably with rapidly urbanizing town, is a town with a dispersive urban growth but lacks major features of an urban settlement such as population size and economic activities.

Sanitation: refers to practices related to adequate management of human faeces, grey water, solid waste, and other environmental wastes which are potentially pathogenic.

Dug well: a tube usually of diameter between 1to 1.5 m and depth between 3-10 m, bored into the ground manually or mechanically to access shallow groundwater, or water at the water table level.

Borehole: water source from deep groundwater.

Wells: these are boreholes and dug wells from which groundwater can be abstracted.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

It appears that not much scientific investigation has been done on the occurrence and concentration of human enteric viruses on ground water particularly under sub-Saharan African slums and low income settlements where a higher toll of pathogen contamination is likely. It is reasonable to presume that this is because aquifers by nature are known to be free of infectious viruses, and also because in the developed world where sanitation is improved, there is less expectation of microbial contamination of ground water and less groundwater extraction without treatment thus little interest in investigating the contamination level due to viruses. Some studies such as Ground et al. (2011) assessed the risk of onsite sanitation to groundwater in the region but failed to support their conclusion with practical results of microbial contamination. In urban poor communities in the developing world where sanitation standards are low, there are tendencies of viral intrusion into groundwater systems owing to the size of viruses and their ability to travel long distance (Krauss & Griebler, 2011; Okoh et al., 2010). Bacteria also could be present in groundwater sources but the concentration of faecal indicator bacteria in groundwater in the region in relation to risk of environmental factors has also not been thoroughly investigated. A study of groundwater quality in an informal settlement of Zimbabwe revealed that two third of boreholes and domestic wells had detectable levels of fecal and total coliforms (Graham & Polizzotto, 2013). Similar works which have been done on virus presence in water sources are summarized below.

A study conducted by Borchardt et al. (2012) on non-disinfected drinking water from taps supplied by municipal wells in 14 Wisconsin communities in the United States revealed

that out of the 1,204 taps, 284 (24%) were positive for at least one virus type and 41 (3%) for at least two virus types. Adenovirus, entero-viruses and norovirus genogroup I (NoV-GI) were the most frequently detected. The study concludes that population served by non-disinfected groundwater may be exposed to acute gastrointestinal illness (AGI). In this study, the glass wool filtration method by Wyn-Jones et al. (2011) was applied for the concentration of the water samples followed by PCR analysis.

Another study was conducted in the United States by Fong et al. (2007) to investigate the cause of a groundwater associated outbreak which affected approximately 1,450 residents and visitors of South Bass Island, Ohio between July and September 2004 groundwater contamination. The result shows that Adenovirus was found in 2 out of the 16 wells sampled.

In sub-Sahara Africa, a similar work was done by Katukiza et al. (2013) on surface water and some shallow ground water sources in Bwaise III, a slum community in Kampala. In that research, at least 70% of the samples tested positive for human Adenovirus. The glass wool filtration protocol of Vilagines et al. (1993) as modified by Wyn-Jones et al. (2011) was applied after tested with samples of bacteriophages PRD1 and φx147 to confirm its effectiveness of recovery of virus particles.

2.2 Channel of pathogen transmission via groundwater

Enteric viruses and bacteria come from human intestines into the environment when human excreta is not properly managed or treated before disposal into the environment. They are the most common contaminant of groundwater (Okoh et al., 2010). They take different routes to get to groundwater. Figure 2.1 shows a flow scheme of major

transmission route of human enteric pathogens in the water phase. Human excreta are transported to groundwater when runoff from precipitation, leaking underground sewage or faecal sludge from pit latrines, and solid waste that contains excreta leaches and infiltrates through permeable soil formation into shallow and deep ground water (Jamieson et al., 2002; Foppen et al., 2007). Pathogens however can also be transmitted through ingestion of contaminated solid food, and shellfish grown in sewage polluted marine environment (Jean et al., 2004; Okoh et al., 2010).

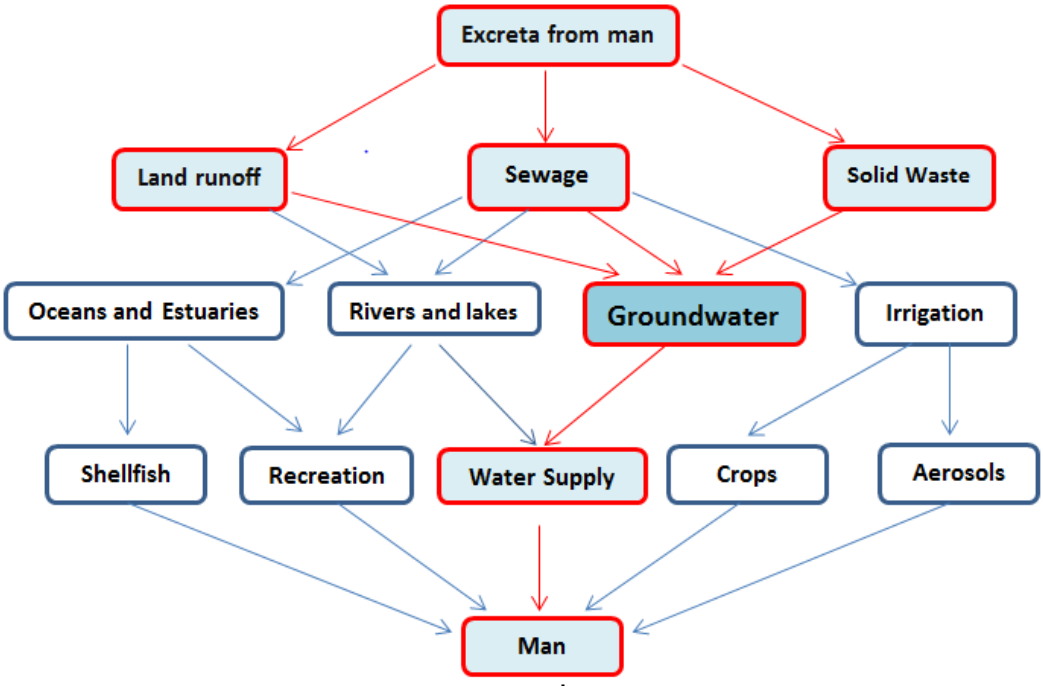


Figure 2.1: Enteric virus transmission routes
(Source: Bosch, 1998)

For bacteria and viruses, the transport mechanisms such as pattern of groundwater movement, interaction with soil condition, particulate flow regimes through pores of soil media which influences the presence of these microbes at various depths of soil strata is a complex phenomenon with scarce information in the literature.

Okoh et al. (2010) observes that the extremely small size of virus particles enables them to flow through soil pores from a source of contamination such as sewage pipes, pit latrines and septic tanks, eventually to shallow dug wells and aquifers. The particles/viruses were found to be capable of flowing with groundwater unto a depth of 67m and horizontally through a distance of up to 400m (Okoh et al., 2010). Their movement in the sub surface also depends on the water saturation state of the soil. In saturated soil, the pores open up. Virus particles thus flow faster. Another environmental factor that influences the transport of microbes over and below ground surface is rainfall which results to spread and percolation of pathogens into groundwater by runoff over land where manure or bio-solids have been applied (Santamaría & Toranzos, 2003).

2.2.1 Common sources of groundwater contamination

Pit Latrines

Pit latrines are unlined dug holes of depth between 2-4m which most times intercept with the water table. They are the most common human excreta disposal system in low income communities and poses serious threat to the quality of nearby shallow wells through leaching of microbes and chemical pollution (Graham & Polizzotto, 2013).

Untreated sewage and run-offs

Sewage water contains over 100 virus species that cause a wide variety of diseases in man. Sludge produced from wastewater treatment plants contains pathogenic organisms such as bacteria, viruses and parasites that are transmitted through the faecal-oral route(Mocé-Llivina et al., 2003). Human enteric viruses can enter the water environment through the discharge of sewage contaminated water (Bosch & Bosch, 1998). Wastewater

from households discharged into open drains or the environment, and surface runoffs that have been in contact with human and animal excreta can easily enter unprotected wells or leach into ground water system. Conventional wastewater treatment is known to be able to remove just about 50% of virus particles in the settling process while the remaining can only be partly removed by disinfection and the rest discharged into water bodies (Okoh et al., 2010).

Dumpsite

Hospital and industrial wastes disposed of in open dumpsites and domestic wastes containing human or animal excreta can leach pathogens into surface and groundwater bodies. In the event of flooding, the transport of these waste materials are enhanced and deposited into wells or permeable soil where they eventually infiltrate into the groundwater system.

Graveyard

Dead body is an organic matter that slowly decomposes, and may be a reservoir of all kinds of pathogens depending on the cause of death. Infiltration of precipitation through loose soil or rise in shallow water table can facilitate leaching of microbes into shallow and deep groundwater especially if they are located within 300 meters at downstream end.

2.3 Sanitary inspection

Sanitary inspection in water quality management is a fundamental activity for safety and sustainability of water supply systems and projects. It is basically an onsite inspection

and evaluation, usually undertaken by qualified individuals, of all conditions, devices and practices in the water supply system that threatens or poses danger to the health and well-being of the consumer (WHO guideline for drinking water quality). The objective usually is to predict current or potential microbial contamination to water sources.

The idea of guided sanitary inspection was initiated by WHO in 1997 which accompanied a checklist of contamination factors. The WHO checklist of risk of contamination (ROC) is widely used and several studies including Vaccari et al. (2010) and Mushi et al. (2012) have succeeded in predicting microbial contamination of water sources by way of sanitary inspection using the ROC scoring. However, the effectiveness of this checklist to predict viral contamination of groundwater is yet to be determined in the absence of such investigation in the region. Another method with checklist of sanitary inspection was proposed by Figueras et al. (2000). Figueras and co-workers identified a systematic process for sanitary inspection and sampling which includes pre-inspection preparation, onsite visit, and sampling location time and frequency planning. The on-site visit constitutes the actual sanitary inspection using the checklist which considers both pathogen and chemical hazards to water sources unlike the WHO adopted guidelines. The choice of method may depend on the objectives of the inspection, type of source, and site condition.

2.4 Contamination risk to water points

Among other factors like geology and hydrogeology, the two major factors that greatly influence the microbial quality of groundwater are the construction of the well and environmental sanitation condition. The risk could result from poor siting associated with

environmental sanitation problems like faecal sludge and grey water management, well condition, usage and distance from a point of contamination (January et al., 2015). Figure 2.12 shows the level of risk to contamination of boreholes and dug wells as influenced by horizontal and vertical distance.

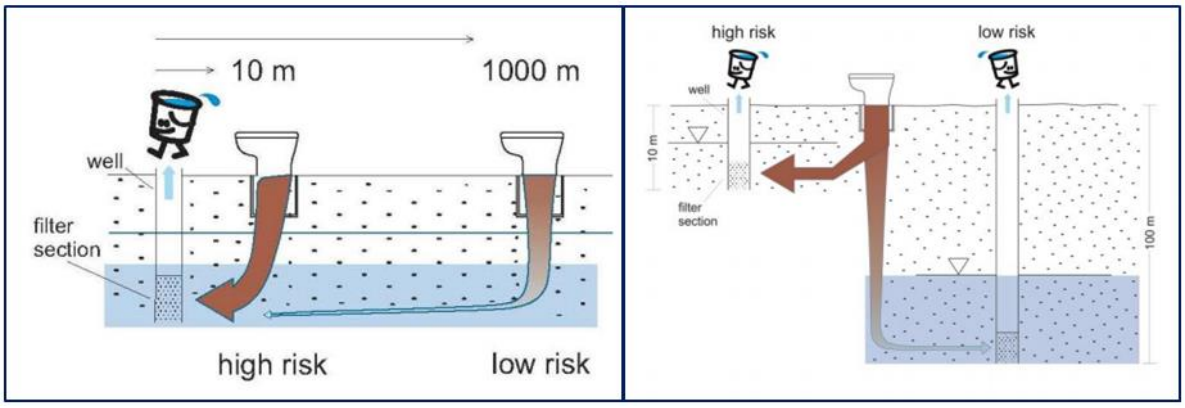


Figure 2.2: Influence of distance from source to contamination risk level of deep and shallow groundwater (Source: Susana Document; How to keep your groundwater drinkable: Safe Siting of Sanitation Systems)

Several approaches such as models, tracers, and physical inspection have been applied to assess contamination of groundwater. Two approaches are available for the assessment of groundwater contamination risk based on Sanitation improvement programs (Affairs, 2003). These include two stage assessment and single stage assessment. Two stage assessments constitute area based hydrological assessment followed by project based sanitation survey. Single stage assessment involves a more detailed project level assessment and is applied where no area based assessment has been carried out. Howard et al. (2003) wrote the Guidelines for Assessing the Risk to groundwater from On-Site Sanitation. They considered 11 contamination risk factors to groundwater (see Appendix 3). These factors and the approach to the assessment are based on physical inspection by

observation. Other possible factors such as sub-surface conditions, hydrogeology, hydro-chemical, and properties of the contaminant were not directly considered.

Specialized groundwater contaminant transport models, both mathematical and software tools have been developed. The parameters involved are mostly generated from site conditions both at surface and sub-surface. This could be a complex and expensive process. Depending on the objective and available budget, a specific model or risk assessment process can be selected. The literature scarcely provides a simplified mathematical model for assessment of the influence of environmental sanitation factors to contamination of dug wells and boreholes.

2.5 Pathogens and their infection pathways in humans

Water-related pathogens are commonly placed under disease category such as gastrointestinal, respiratory or skin conditions. Figure 2.3 shows the transmission pathways for, and examples of water-related pathogens. Human enteric bacteria and viruses (rotavirus and adenovirus) which are the focus of this research are contacted by ingestion (drinking), or inhalation and aspiration (of aerosols) mostly during bathing with contaminated water. They are not known to cause skin infections. However, adenovirus is known to be a cause of eye infection (conjunctivitis) in addition to respiratory diseases.

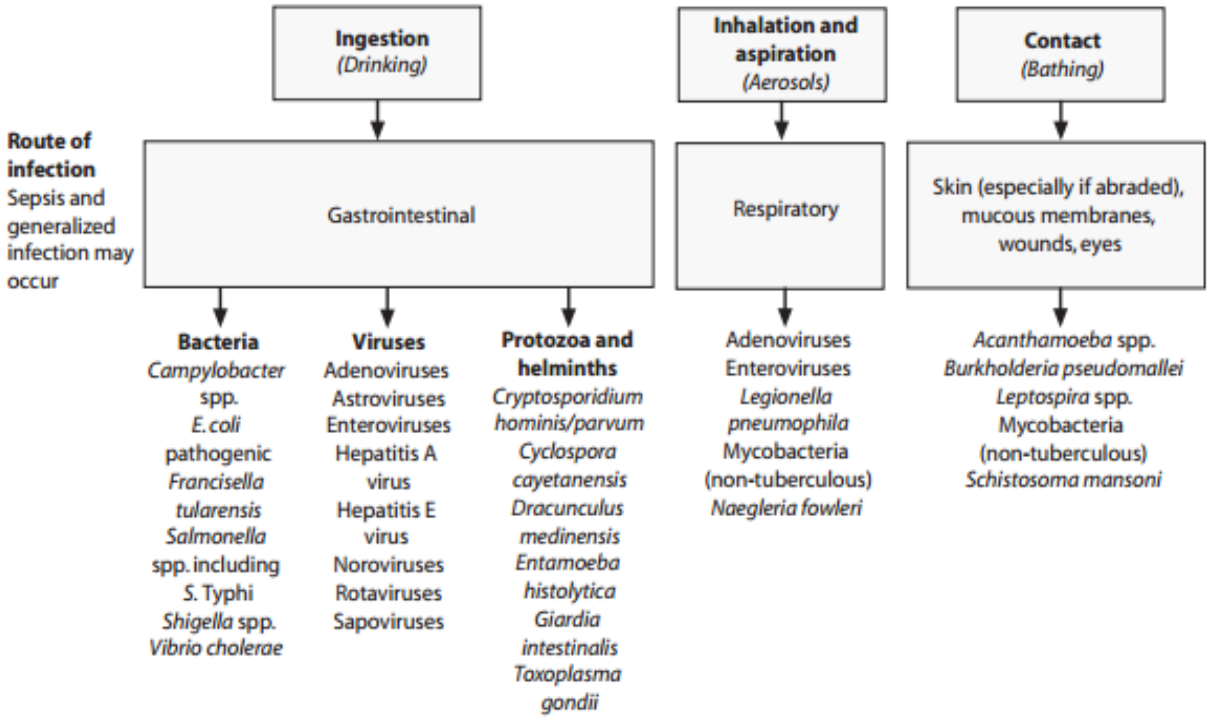


Figure 2.3: Transmission pathways for, and examples of water-related pathogens
Source: WHO guidelines for drinking water, fourth edition (2011)

2.6 Viruses

2.6.1 Nature and classification

Nature

Viruses are minute obligate intracellular parasites (Schaechter et al., 2007). Their sizes range between 0.025-0.3 μm. In relation to other organisms, they occupy the transition zone that separates the living from the non-living. They contain either RNA or DNA genome surrounded by a protective, virus-coded protein coat (Ding, 2008). Even though their basic structure is made up of nucleic acid and a protein coat, they do lack the enzyme necessary for the synthesis of nucleic acid and protein. They have to hijack the synthetic machinery of the host cell in order to replicate nucleic acid and protein to

produce more virus cells (Ding, 2008; D. Roy Cullimore, Practical Manual of Groundwater).

Viruses cause infection in plants, bacteria, humans and other animals and they are specific for the host (or have a narrow host range). Their medical importance stems from the fact that they are very infective and can cause a very wide range of human diseases from minor ailments like cold to terrifying diseases like HIV and EVD (Ebola Virus Disease). Most viruses are inactivated by heat at temperatures above 56^{o}C. Figures 2.4 show the basic structure of virus and the location of their nucleic acids.

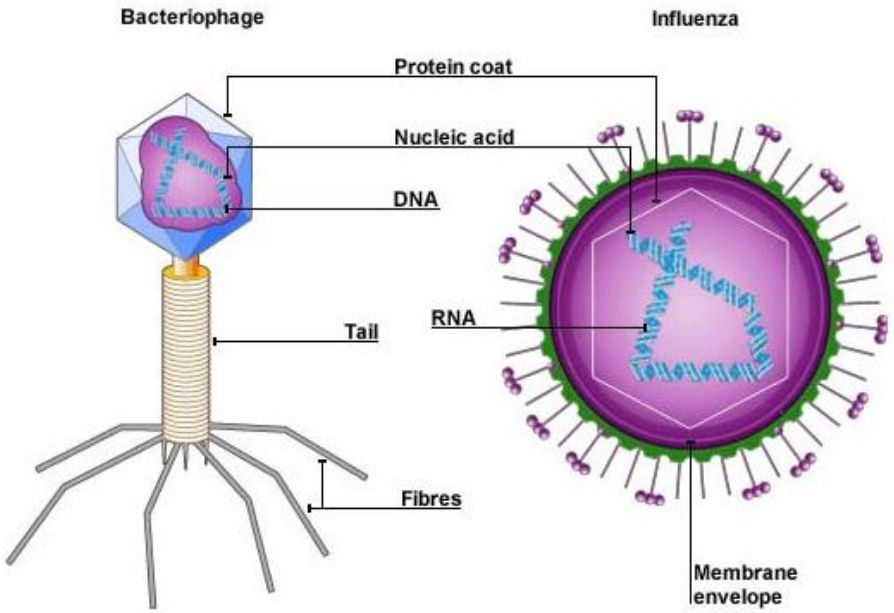


Figure 2.4: Basic structure of Bacteriophage and influenza viruses showing nucleic acids and protein coat
(Source: online images for viruses: BBC science and nature)

Classification

Classification of viruses starts at the level of order through family, genus and species (i.e., they do not belong to any kingdom, phylum or class of living things). The

classification is mainly based on their phenotypic characteristics such as: nucleic acid type (DNA or RNA), morphology, mode of replication (e.g. retrovirus), the host they infect (animal, plant, bacteria), and the disease they cause (e.g. hepatitis virus) (Fauquet et al., 2005). There are currently two classification schemes: the International Committee of Taxonomy of Viruses (ICTV) and the Baltimore classification system (wikipedia.com). The two systems are however complementary in application. According to ICTV (the body charged with the responsibility for classification of viruses), so far, 7 orders, 96 families, 22 sub-families, 420 genera, and 2,618 species have been classified. However, the majority of virus families are yet to be classified. The most common classifications are based on their chemical composition (including mode of replication in some cases), and their morphology.

Chemical composition: this refers to the genomic constitution, i.e., DNA or RNA virus. The nucleic acid molecule(s) may be single stranded or double stranded, linear or circular in shape. The viral nucleic acid (genome) contains the genetic material necessary for replication and each of these genomes necessitates different replication strategies. Some viruses have membrane, others do not. Some DNA viruses include: Adenovirus, Pavovirus, Herpesvirus, Bacteriophage, and Poxvirus. Some RNA viruses include: Rotavirus, Norovirus, Reovirus and the Retroviruses. Figures 2.6 and 2.7 shows the classes of virus based on their chemical composition.

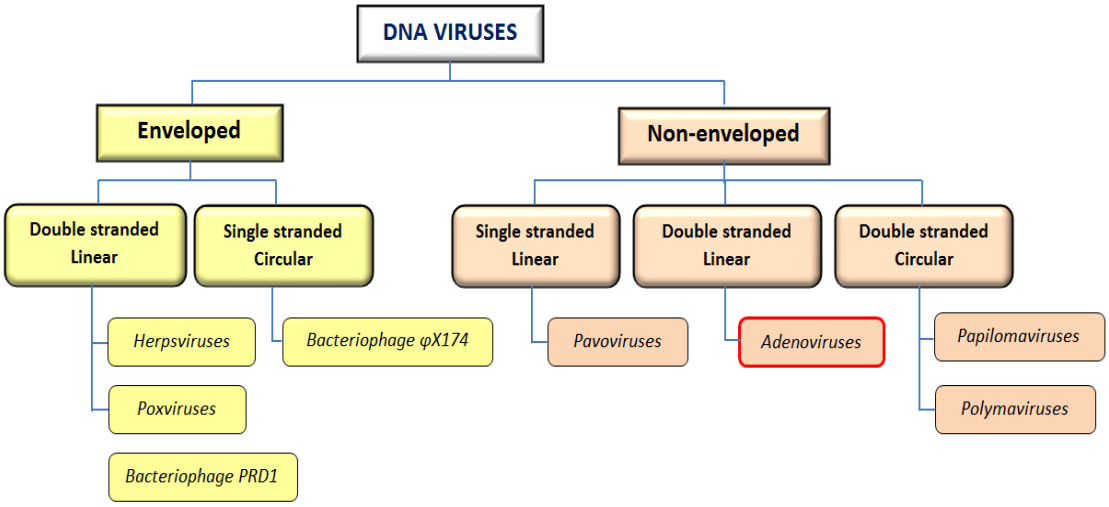


Figure 2.5: DNA virus classes
(Source: Schaechter et al., 2007)

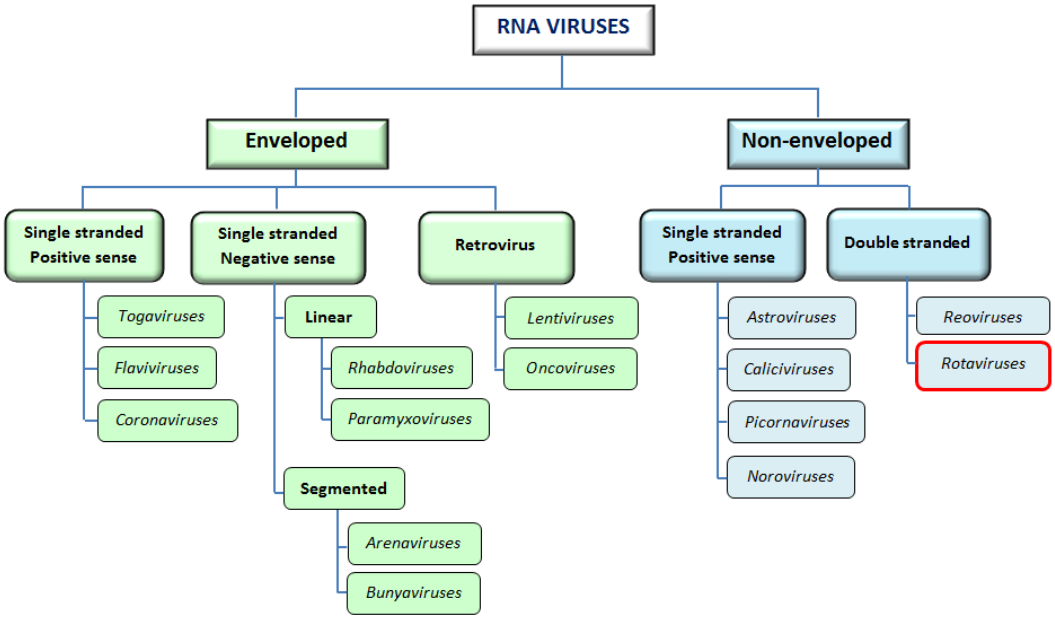


Figure 2.6: RNA virus classes
(Source: Schaechter et al., 2007)

Morphology: this is about the shape of the outer envelope or capsid that protects the protein coat. There are three types: icosahedral morphology, helical morphology, and complex symmetry. *Icosahedral morphology* is typical of the nucleocapsids of most spherically shaped viruses. The head consists of a regular polyhedron with 20 faces.

Bacteriophage is an example of a virus with a *complex symmetry*. Many viruses have capsid or envelope while a few lacks it.

Helical morphology is typical in the nucleocapsids of most pleomorphic and filamentous viruses. Helical nucleocapsids consist of a helical array of capsid protein called protomers, wrapped around a helical filament of nucleic acid, e.g. Tobacco mosaic virus.

2.6.2 Common viruses in groundwater

Groundwater viruses include enteroviruses and human enteric viruses such as rotavirus, adenovirus, and hepatitis A and E virus. Table 2.2 shows common waterborne viruses found in groundwater, the diseases they are responsible for and their source of entry into the environment.

Table 2.1: Viruses found in groundwater

(Source: Krauss & Griebler, 2011)

Virus	Major Diseases	Source
Poliovirus	Poliomyelitis	Human faeces
coxsackievirus	Fever, pharyngitis, respiratory diseases, diarrhoea, hemorrhagic conjunctivitis, myocarditis, pericarditis, aseptic meningitis, encephalitis, reactive insulin-dependent diabetes	Human faeces
Rotavirus A and C	Gastroenteritis	Human faeces
Adenovirus	Respiratory disease, gastroenteritis	Human faeces
Coronavirus	Gastroenteritis	Human faeces
Echovirus	Respiratory diseases, aseptic meningitis, rash, fever	Human faeces
Enteroviruses 68-71	Polio-like illness, aseptic meningitis, hand, foot and mouth (E71), epidemic conjunctivitis (E70)	Human faeces
Hepatitis A	Fever, nausea, jaundice, liver failure	Human faeces
Hepatitis E	Fever, nausea, jaundice	Human faeces
Norovirus (Norwalk	Gastroenteritis	Human faeces

virus)		
Calicivirus	Gastroenteritis	Human faeces
Astrovirus	Gastroenteritis	Human faeces
Sapovirus	Gastroenteritis	Human faeces
Orthoreovirus	Gastrointestinal and upper respiratory disease	Human faeces

2.6.2.1 Enteric viruses

These are animal viruses that live and replicate in the intestinal epithelium of animals, and those that first multiply in the intestine and then spread to extra-intestinal target organs where they cause other diseases. They are leading causes of nonbacterial gastrointestinal illness worldwide (Parshionikar et al., 2010; Logan et al., 2006). An infected individual could excrete between 10^5 and 10^{11} virus particles per gram of stool (Okoh et al., 2010). Enteric viruses can also cause meningitis, respiratory infections, conjunctivitis, encephalitis, and paralysis, and can range from mid to life-threatening illness (Parshionikar et al., 2010; Bosch and Bosch, 1998). The enteric viruses transmitted through water may include *enteroviruses*, such as poliovirus, coxsackie virus, and echovirus; *human caliciviruses*, such as noroviruses (NoV) and sapoviruses; *rotaviruses*; *hepatitis A virus* (HAV); and *adenoviruses* (Cashdollar et al., 2013). The transmission of these viruses occurs through the feaco-oral route via ingestion of contaminated food or water and also by direct contact with an infected person.

Human enteric viruses can also be transmitted through non liquid food substances (Okoh et al., 2010; Jean et al., 2004). Their infectious dose is also very low with one particle capable of igniting an infection (Katukiza et al., 2013). Waterborne human enteric viruses include rotavirus which is one of the causers of gastroenteritis; enterovirus (appearing as

poliovirus, coxsackievirus, and echovirus) which is the cause of paralysis, meningitis, respiratory diseases, hand-to foot and mouth disease, heart anomalies, pleurodynia, and gastroenteritis; coronavirus which also causes gastroenteritis and respiratory diseases; and hepatovirus which is the cause of hepatitis A, C, D, E and F.

Rotavirus

Rotavirus is a triple coated (three layered capsid), double stranded RNA virus belonging to the family Reoviridae (Chin, 2000). Rotavirus infection causes diarrhoea or gastroenteritis in children worldwide (Gratacap-Cavallier et al., 2000; Chin, 2000). There is no known cure for rotavirus infections except for its prevention through vaccination (Okoh et al., 2010). Figure 2.8 shows a schematic representation of rotavirus showing its triple layered protein coating: an outer capsid (VP7), an inner capsid (VP6), and an inner core (VP2) that surrounds the virus' 11 segments of double-stranded RNA (Dennehy, 2008).

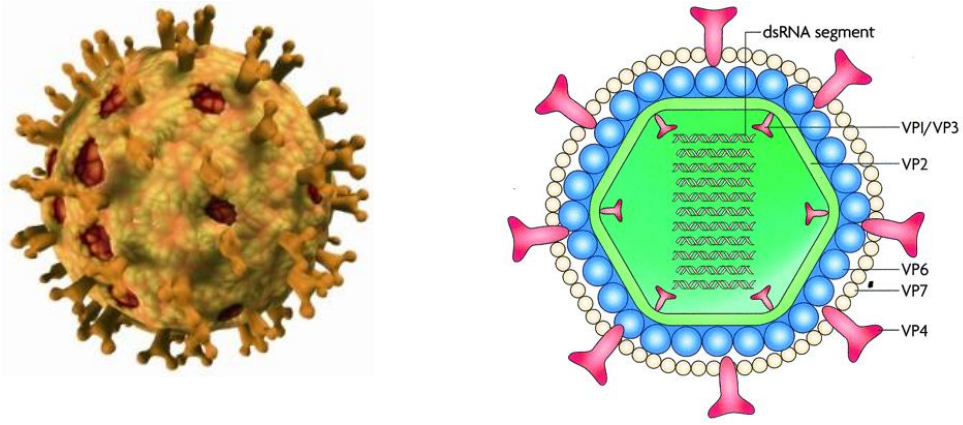


Figure 2.7: Schematic representation of Rotavirus
(Source: www.cofa.org.ar; Dennehy, 2008)

Adenovirus

Adenoviruses (AdV) are large naked or non-enveloped viruses with characteristic fibres projecting from each vortex of their icosahedral capsids. Their genome is double stranded DNA of 34-38kb, and with an icosahedral capsid (Okoh et al., 2010). They are cause of a wide variety of diseases in human including pharyngitis, respiratory diseases such as killer cold and pneumonia, hemorrhagic cystitis, gastroenteritis, and keratoconjunctivitis. They are also known to be the cause of some life-threatening opportunistic infections in immune-compromised individuals and are responsible for outbreaks in certain populations (Buckwalter et al., 2012). Based on its biological properties, Adenovirus are classified into six species (A to F), and a total of 51 serotypes (Okoh et al., 2010). Figure 2.9 shows a three-dimensional, and a schematic representation of the structure of adenovirus showing its vortices, capsids and nucleic acid.

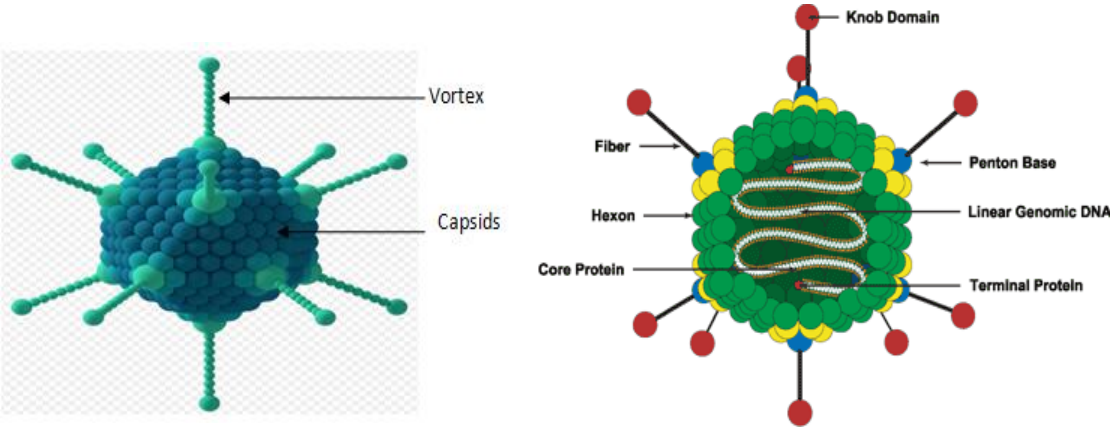


Figure2.8: Outer structure and schematic of Adenovirus
(Source: commons.wikimedia.org)

Norovirus

Human norovirus is a positive sense single-stranded RNA virus of about 7.7 kb belonging to the genera Norovirus and the family Caliciviridae (Patel et al., 2008). They are the

second most common cause of gastroenteritis of viral origin worldwide (Parshionikar et al., 2010; Manuel et al., 2015). In industrialized countries, they are considered to be the most common cause of acute non-bacterial gastroenteritis in both adults and children. In developed countries like United State where sanitation is improved, norovirus accounts for 19 million to 21 million illnesses annually (Manuel et al., 2015). Figure 2.9 shows the basic structure of norovirus.

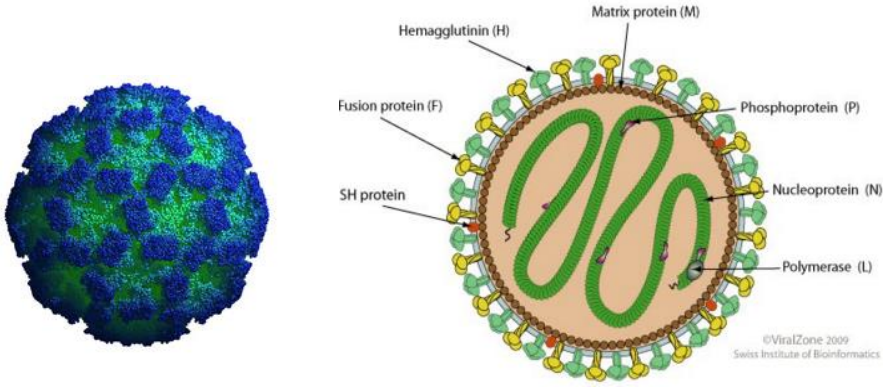


Figure 2.9: Structure of Norovirus
(Source: Brown, 2012)

2.7 Virus detection and quantitation

Two methods are currently known for identification and characterization of microbes, the *culture* method and *molecular diagnostic* method. Unlike bacteria, it is impossible to identify and quantify viruses using traditional culture methods. This is because viruses cannot metabolize or replicate by themselves (Ding, 2008). They cannot be grown in standard microbiological broths or an agar plate. They thus have to be cultured inside suitable host cell (plaque assay), a requirement that complicates the detection, identification, and characterization of viruses (Ding, 2008). In the culture method, three

types of media have thus been developed by virologists: culturing viruses in bacteria, in plants and animals, or in embryonated chicken eggs.

However, molecular diagnostic method which applies manipulation of DNA/RNA or protein has revolutionized viral metagenomics by shortening the detection time and increasing the accuracy of detection (Mackay et al., 2002). It is therefore the method currently widely applied in viral metagenomics. The processes and other involvements in viral genome detection and quantification using molecular diagnostic approach are given in subsequent sections.

2.7.1 Methods for concentration of water sample for virus detection

Sample concentration is the key first step in evaluating pathogen levels in suspected contaminated water (Millen et al., 2012). However, the chances of acquiring virus particles on groundwater depend on the level and source of contamination. For surface water, sample quantity of 10-70 litres is adequate for concentration. Because of the much lower concentration of virus particles in ground water, it will be advantageous for the sample quantity to be much higher to increase the probability of capturing virus particles. Several groundwater concentration methods have been developed and modified to enhance their efficiency. Specific concentration methods tend to be applicable for a specific pathogen group (Millen et al., 2012). The choice of method depends on the type of project with respect to sample quantity and size, and available budget. Common virus concentration methods include Glass wool filtration, hydro-extraction, Adsorption technique which utilizes the charge properties of virus by adsorption on negatively charged surfaces, ultrafiltration technique which involves filtering water through

membranes or hollow fibres, and ultracentrifugation technique which is not too applicable for large volume samples (Wyn-Jones & Sellwood, 2001; Percival & Wyn-Jones, 2013). Wyn-Jones and co-worker Sellwood proposed the following as the basic criteria for ideal concentration method in their review.

i. Should be simple to use and operates faster
ii. Should be capable of high virus recovery rate
iii. Should be capable of filtering large amount of viruses
iv. Should give a little volume of concentrate
v. Should be able to filter large volume of water
vi. Should be repeatable and reproducible
vii. Should be cheap

2.7.1.1 Glass wool filtration

Available filtration methods for concentration of virus particles on groundwater samples are either too costly for studies requiring large number of samples, limited to small sample volumes, or not very potable to undertake routine field applications (Lambertini et al., 2008). A study to evaluate the performance of glass wool filters undertaken by Lambertini et al. (2008) proved successful in concentrating the four viruses on the U.S. Environmental Protection Agency contaminant candidate list, i.e., coxsackievirus, echovirus, norovirus, and adenovirus, as well as poliovirus in municipal wells in Wisconsin.

The glass wool method was first developed by Vilagines et al. (1993). It was modified by Wyn-Jones et al. (2011). It is based on the principle of adsorption of virus particles on

glass wool. Glass wool has a relative density of 2.5-2.6. It is an amorphous silicate material produced from glass fibres, secured with binders and oiled for dust suppression (Shahrizal & Razak, 2011).

Virus charge is categorized by an isoelectric point at which the charge of the particle is zero (Katukiza et al., 2013). Virus adsorbs exclusively to negatively charged silica at pH values below their isoelectric points, i.e., under conditions favoring a positive surface charge on the virions (Zerda et al., 1985). The sample water to be concentrated is conditioned to pH of around 3.5 with 1N acid. This is to enable adsorption of virus particles onto glass wool. The mechanism is that when water at low pH passes through glass-wool, the glass-wool material becomes positively charged. Virus particles are negatively charged and can therefore easily adsorb to the glass wool material due to electromagnetic attraction.

Adsorbed virus particles are then eluted with beef extract (in glycine buffer) at pH 9.5. The purpose of this process was to detach virus particles from the glass-wool. The mechanism is by electrochemical desorption, i.e., at high pH, the virus particles becomes negatively charged, and are therefore released from negatively charged glass wool. Afterwards the eluted sample is organically flocculated at pH 3.5. It is then centrifuged at 3750 – 4000 rpm and the pellets re-suspended with Phosphate Buffered Saline at pH 7.

This application of this method in this research could be feasible due to the following reasons:

I. It has been tested and proven effective in concentrating groundwater samples in previous studies such as in Katukiza et al., 2013.

II. It is affordable, simple to assemble the setup and be operated.

2.7.2 Nucleic Acid Extraction from virus particles

The purpose of extracting nucleic acids from virus particles is to make them available as the template DNA for the qPCR process. Rotavirus and Norovirus are RNA viruses while Norovirus is a DNA virus. The extraction of RNA is normally critical due to the free abundance of the enzyme RNAse which has the tendency of degrading RNA into protein, and which is very difficult to inactivate. The nucleic acid extraction thus needs to be done in a clean DNA lab under controlled conditions.

After extraction of the nucleic acids from the sample, if it is a DNA virus, it can be directly run on PCR. If it is RNA, a complementary DNA (cDNA) will have to be synthesized by reverse transcription (RT-PCR) because the PCR process can only amplify DNA segments.

Depending on sample type and choice of application, there are different protocols for DNA/RNA extraction. One applicable protocol for extraction of virus DNA is described by Boom et al. (1999). The method is based on binding of nucleic acid to silica particles in the presence of a high molarity solution of guanidinium isothiocyanate (GuSCN), which is a chaotropic agent. The extracted nucleic acid samples will then be stored at a temperature of -72^{o}C.

2.7.2.1 Nucleic Acids concentration and Purity

The concentration and purity of the isolated nucleic acids from the sample is critical to the success of downstream processes such as quantitation and genome sequencing process (Boesenberg-Smith et al., 2012). A common method normally applied to measure

sample DNA concentration and purity is the ultraviolet spectrophotometry method. It works on the principle of absorption of ultraviolet rays by the bases of a DNA strand. Because each DNA strand has a unique arrangement of nucleotides that forms the bases, the absorption rate will therefore vary with DNA type. The instrument is called NanoDrop spectrometer. It takes measurements at wavelengths of 260 and 280 nm, and in addition determines an absorption spectrum from 220 – 350 nm which is an adequate wavelength band to measure the UV absorption intensity of a reasonable quantity of DNA or protein (Brescia, 2012).

Double stranded DNA concentration is measured in ug/ml according to the relationship:

$$1\ OD_{260} unit = 50\ \mu g/ml$$

Where OD_{260} is the optical density value at wavelength of 260nm

The concentration of single stranded DNA and RNA can be measured by measuring adsorption at wavelength of 260nm. The purity of DNA or RNA is measured as the ratio of their absorption value at wavelength of 260nm over that at wavelength of 280. The A260/A280 is the most widely used method though not actually robust. The NanoDrop spectrophotometer calculates concentrations and purity automatically in ng/µl.

There is a protocol for the application of this method in the laboratory. The procedure leads to the generation of graphs using a software program. The optical density value (OD) and the Absorption values at wavelengths 260 and 280 can be read from the graph to determine the concentration and purity of the nucleic acid.

2.7.3 DNA amplification

2.7.3.1 Polymerase Chain Reaction (PCR)

In molecular diagnosis for viral detection, DNA isolation and PCR analysis are the most important steps in the process. This technique represents a significant practical improvement in the field of molecular biology particularly in the analysis of DNA or RNA (Powledge, 2004; Gibbs, 1991). Virus do not replicate on their own neither can be cultured. PCR is the technique used to produce multiple copies of a segment DNA of interest from a small initial sample. The multiple copies formed provides enough quantity for the detection of pathogenic viruses or bacteria, identification of individuals (DNA fingerprinting), and other scientific research that involves manipulation of DNA or RNA (Powledge, 2004). The discovery of this technique in 1983 by Kary Mullis has opened a gateway of discoveries into the world of microorganisms. Its application in viral DNA analysis has enhanced some important and critical understanding of viruses, which are very significant target for studies in life science due to their infective nature. PCR is based on three consecutive steps that are conditioned under specific temperature levels for the entire DNA synthesis process (Delidow et al., 1993):

1. Denaturing of the template into two separate single strands. It takes place in about 1 minute at a temperature of 94^{o}C
2. Annealing of primers to each of the separated strands (forward and backward primers) for synthesis of a new complete strand. It lasts for about 45 seconds at around 54^{o}C.
3. Extension of new DNA strands from the primers at 72^{o}C within a period of two minutes.

Newly formed pairs of DNA strands repeatedly undergo the three-stage process thereby doubling the amount of target DNA in each step, producing multiple copies of DNA.

Under ideal conditions, the PCR amplification process proceeds according to the formula:

$$A = B(1 + e)^n$$

Where:

A = amplified products, B = template DNA, n = cycle number, and e = amplification efficiency.

2.7.3.2 Quantitative PCR (qPCR)

Most of the diagnostic PCR assays developed earlier have been applied in a qualitative, or 'yes/no' format (Mackay et al., 2002). The RT-qPCR is an advancement of the PCR (traditional endpoint PCR) as it undertakes quantitation of target nucleic acid right inside a diagnostic laboratory. In the traditional endpoint PCR, estimation of the amount of synthesized DNA is made at the end of the reaction (plateau phase). In qPCR, quantitation is at the end of each PCR cycle and not at the end in the plateau phase (Mackay et al., 2002). This provides the flexibility of quantitating the difference between each cycle start amount of DNA for each sample. It also has the advantage of decreasing variability in results as well as the ability to achieve a high productivity of the samples analyzed. Considering the tendency of concentrating a very small number of virus particles from groundwater and subsequently the isolation of less substantial quantity of DNA, quantitative Polymerase Chain Reaction (qPCR) is a technology applied whereby the number of cycles necessary to detect and quantify DNA copies is monitored and acquired.

Several researches including Katukiza et al. (2013), and Heim et al. (2003) have successfully applied qPCR to amplify human adenovirus or other human enteric and waterborne viruses. Mackay et al. (2002) observed that the disadvantage of using qPCR as compared to traditional PCR include the inability to monitor the size of an amplicon without opening the system, the incompatibility of some platforms with some fluorogenic chemistries, and the relatively restricted multiplex capabilities of current applications. Quantitative PCR (qPCR) assay has the same basic ingredients as the regular PCR. However, an additional component, *fluorescent probe*, is added to the sample ingredients of the traditional PCR. The probe facilitates quantitative estimation of amplified DNA at each cycle which is thus referred to as quantitative PCR. The probe can only bind to double stranded DNA, which quantity is doubled after each step. The common ingredients are as follows:

1. **Water media** to provide the matrix.
2. **Buffer (10 x PCR):** creates an enabling environment for optimum activity for Taq DNA polymerase by maintaining a stable pH and salt condition.
3. **Template/target DNA** to be amplified.
4. **Primers (forward and reverse):** a synthetic single-strand DNA sequence designed to locate the target DNA fragment.
5. **dNTPs mix:** mix of nucleotides which are the building blocks of new DNA strand.
6. **Taq polymerase:** an enzyme that helps catalize the polymerisation of the deoxynucleotide for synthesis of new DNA.

7. **25mM $MgCl_2$:** for primers and DNA stabilization and for optimal activity of the Taq DNA polymerase enzyme.

2.7.3.3 Reverse Transcription qPCR

Unlike DNA, RNA viruses cannot be directly recognized and amplified by the PCR process. For this to happen, the RNA must first be converted enzymatically to copy-DNA (cDNA) through an additional primary reaction process (reverse transcription) mediated by an RNA-dependent DNA polymerase enzyme, *reverse transcriptase* (Bustin and Mueller, 2005). Several studies have indicated that Reverse Transcript-PCR can be used for the simultaneous detection of RNA of various viruses.

Researches have applied two protocols for the conversion of RNA to cDNA: the one tube RT-PCR assay, and the two-tube assay. In the two tube assay, the reverse-transcription reaction and PCR are performed sequentially in two separate reaction tubes using an arbitrary oligo-dT or sequence specific primer. In the one-tube assay, both reactions are performed in the same tube using a single buffer in the presence of high concentration of dNTPs and either target specific or oligo-DT primers (QIAgen, 1999; Bustin and Mueller, 2005; Katukiza et al., 2013). The one-tube assay protocol is more effective for amplification of targets of reasonable quantity at the start of the reaction and is more convenient as it reduces the risk of cross-contamination and RNA degradation as suggested by (Bustin 2005). For successful reproducibility of cDNA in the RT-PCR, the quality of the RNA template is the most critical factor (Bustin, 2005; Monti, 2011). It is therefore important that RNase is excluded or inhibited during the RNA extraction process. For this reason, adequate and very strict measures such as clean working area

and constant wearing of gloves must be followed. The following factors affect amplification efficiency in the RT-PCR process (Siebert, 1999):

1. the efficiency of reverse transcription
2. Mg2+/ dNTPs/ primer concentrations
3. enzyme activity
4. pH
5. annealing temperature
6. temperature variation
7. cycle number and
8. tube to tube variation

2.7.4 qPCR Assay

There are two types of fluorescent assays commonly applied in qPCR: SYBR green, and probe (TaqMan) based. SYBR green is a fluorescent intercalating dye. Its mechanism of action is that the dye attaches to every newly synthesized DNA strand and in the process gives out a fluorescent energy which is used as a signal after the completion of each cycle. The signals after each cycle are recorded by the qPCR instrument. The fluorescence signal strength recorded is directly proportional to the total number of DNA strands synthesized during the PCR process. One of the shortcomings of the application of intercalating dye assay is its lack of specificity that may result to false estimation of initial copy number (Brasileira et al., 2013). SYBR's intercalator dye detects accumulation of all double stranded, i.e. both specific and non-specific, PCR products. The non-specific products are actually not wanted. This disadvantage of SYBR (production of unwanted products) is however addressed in the TaqMan chemistry. Unlike SYBR green,TaqMan uses a fluorogenic probe specific to target gene thus making

it possible to measure only specific PCR products. SYBR green has the advantage of low cost but does not allow multiplexing or genotyping. Probe assays though relatively expensive, allows genotyping and has high level quantitation and high reproducibility. SYBR green has the disadvantage of melting point curve determination unlike probe assay like 5' hydrolysis. The type of fluorescent assay applied defines the technique used as either SYBR green or probe based technique.

2.7.5 Nested PCR

PCR-based methods for gene analysis have been widely applied in the study of microbial diversity. However, the analysis would be difficult when the DNA content in samples is too low to be amplified by conventional PCR method (Fan et al., 2009; Haff, 1994). The nested PCR is a variation of the traditional PCR method in which two instead of one pair of primers are used to amplify a DNA fragment (Fan et al., 2009). Consequently, two separate runs of PCR takes place immediately after another. The first set of primers amplifies a fragment just as in the standard PCR. However, a second pair of primers called nested primers (as they lie or are nested within the first fragment) bind inside the first PCR product fragment to allow amplification of a second PCR product which is shorter in length than the first one (Fan et al., 2009). The process scheme is illustrated in figure 2.10.

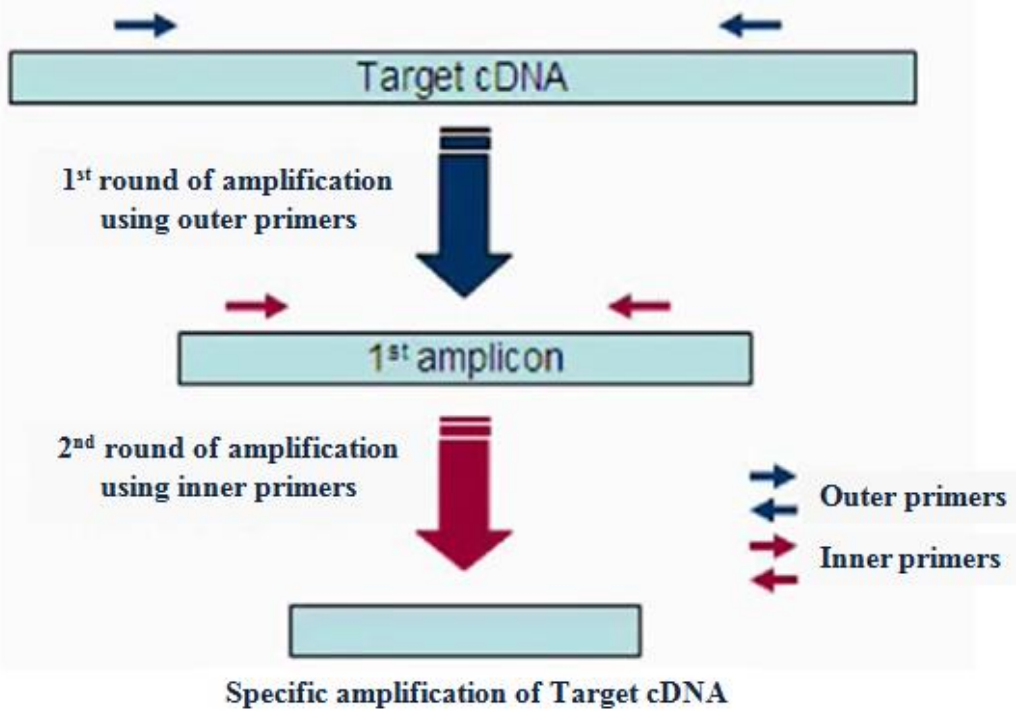


Figure2.10: Schematic of Nested PCR process

The Nested PCR method has two advantages over the conventional PCR:

i. Specificity: because two different sets of primers are used, specificity is increased and the amplification of contaminants decreases because if the wrong PCR fragment was amplified, the probability that the region would be amplified a second time by the second set of primer is very low. Thus nested PCR is a very specific PCR amplification method.

ii. Sensitivity: nested PCR can be even more sensitive than the normal PCR process depending on the number of amplification cycles used in each PCR step. It is that sensitivity that allows nested PCR to amplify very low target concentration.

The chances of detecting virus particles from groundwater as compared to surface water are not too high. Therefore, the sensitivity of the qPCR process to amplify nucleic acid from groundwater could be low. The discriminatory power of the primers in the nested PCR method is so high that a single amplification product can often be obtained with low target concentration (Brasileira et al., 2013). Rigotto et al. (2005) tested three PCR methods for the detection of adenovirus associated with cultivated oysters: conventional PCR, nester-PCR, and integrated cell culture PCR (ICC/PCR). The result showed that the nested PCR method was more sensitive than the other two methods.

2.7.6 Inhibition of PCR

The application of real time qPCR technique in DNA forensic analysis has brought significant insight in understanding the nature of viruses (and their identification and quantification) which generally have a very minute nucleic acid amount. However, its accuracy is threatened due to inhibition by foreign substances that may be present in the sample to be analyzed (Bessetti, 2007; Warren, 2012). Inhibitors are substances that interfere with the DNA amplification process such that the process is prevented from completion (Bessetti, 2007). Inhibitors can cause failure of qPCR process even with the availability of adequate DNA template because they alter the activity of reverse transcriptase (RT) and the thermo-stable DNA polymerase enzyme used in the PCR method (Gallup et al., 2010).

A number of researches on qPCR have identified various types of inhibitors which are categorized as either intrinsic or extrinsic chemical inhibitors. In his review of PCR inhibition, Warren (2012) mentioned the following as inhibitors: humic compounds

(natural soil and water sediments), porphyrine residues, myoglobin (muscle), collagen type 1 (tissues), excessive DNA or DNA template, polysaccharides (plant materials, faeces), heavy metals, hematin (blood), urea (urine), melanin (hair), proteinases (milk), indigo dye (blue jeans).

2.7.6.1 Influence of inhibitors in ground water samples

In soil environment, the most common inhibitors are humic acids and fulvic acids which are together referred to as humic substances. In their research, Matheson et al. (2010) discover that 100% (13 samples) of DNA polymerase tested for sensitivity to humic substances exhibited inhibition by varying concentrations of humic acid and 38.4% (5 samples) exhibited inhibition by varying concentrations of fulvic acid. Groundwater is associated with the soil condition of the source location. Soil and water sediments are believed to be the major carriers of humic compounds. The minute amount of viral DNA/RNA amidst potential inhibition by humic substances in water samples could limit successful amplification of virus DNA/RNA from water samples. This is because humic acid can still show up in the extracted DNA sample. As small as 0.08ug/ml of humic acid is sufficient to inhibit the effectiveness of Taq DNA polymerase (Warren, 2012).

2.7.6.2 Inhibition (false negative) prevention

To successfully amplify virus DNA, it may be necessary to prevent or treat extracted DNA sample against inhibition prior to running qPCR process. Studies have shown that if inhibitors cannot be removed physically (by column or reagent based methods), the effect on quantitation can be reduced by series dilution process. Katukiza et al. (2013) realized that they successfully eliminated inhibition after 10 – 100 times dilution from a groundwater sample out of four that showed inhibition of the PCR process. Serial dilution

however has its shortcomings. You get dilution of template DNA/RNA too, so the template concentration can go under detection limit. In an effort to counter these shortcomings, Gallup et al. (2010) invented the PREXEL-Q software program which automates the process of calculating non-inhibitory dilution. They applied the SPUD assay which is a universal system for undertaking rapid quality control (detection of inhibitors) of nucleic acid samples (Nolan et al., 2006). In their experiment, each PCR reaction was spiked with SPUD amplicons or SPUD amplicon-containing plasmids (a SPUD assay used to check for inhibition in each PREXEL-Q designed qPCR reaction). They revealed that all reaction samples were completely free from inhibitors thus concluding that the PREXEL-Q samples and standard dilution calculations can prevent qPCR inhibition. PCR inhibition can be detected by applying spike control measure as described in 2.7.7 below. However, inhibition may also be dependent on qPCR mix and concentration of target nucleic acid.

2.7.7 DNA Extraction Control

Full attainment of the attributes of qPCR technique (which includes high sensitivity, accuracy, and reliability) depends on two factors: template quality and the presence of inhibitory components. To determine whether the sample contains inhibitory components, a process referred to as ‘spike control’ can be done. Spike control is the addition of a known amount of a casual organism’s DNA to the DNA sample to be amplified in order to check for the presence or potential of PCR inhibition (Anon, 2002). To ensure a reliable and accurate quantification of virus nucleic acids in groundwater with potentially less materials, it could be necessary to incorporate or “spike-in” a foreign

DNA to detect interference to the amplification process. The possibility of false negative or positive will be alleviated by this way.

2.8 Bacteria

2.8.1 Classification and Structure

Bacteria are prokaryotic microorganisms that constitute one of the three domains of the phylogenetic division of life proposed by Woese et al. (1990) based on molecular properties of life forms. The other two domains are Archaea and Eukaryota. Below the domain level are the kingdoms. Kingdoms under the bacteria domain include cyanobacteria, gram positive, green filamentous bacteria, etc. Advancement in molecular diagnostic techniques such as sequencing provides a breakthrough on the diversity of the world of bacteria and giving rise to the sequencing of several thousands of prokaryotic genomes (Schleifer, 2009; Gupta & Sharma, 2015). This approach has proven to be highly effective in providing an insight to the structure of bacterial genomes and the biology and evolution of bacteria (Chan, 2003). Largely, bacteria are classified according to their physical properties and morphology. They can be distinguished by the nature of their cell walls, by their shape, or by the differences in their genetic makeup. These including their growth characteristics are used to detect and identify bacteria in the laboratory. The human system is a host of bacterial cells larger than the number of cells in a human body (approx. $10x10^{12}$ human cells vs. $100x10^{12}$ bacterial cells in human body) (Dutch National institute for Public Health and the Environment, 2014). The majority of bacteria are however harmless and in fact useful to some metabolic processes. They live and survive in specific areas of the body, particularly in the intestines. Some

species are pathogenic, causing diseases like cholera, typhoid fever and bacillary discentry. Unlike viruses, human intestinal bacteria cannot survive outside a host for a long time. They do not grow in the water medium and survives for short periods than virus or protozoa (Gorchev & Ozolins, 2011). This is as a result of their complex growth requirements such as nutrients, carbon and energy for synthesis of cell materials and enzymes. In the absence of some limiting nutrients, they die out easily. Therefore, their presence in water most times indicates recent faecal contamination. However, some bacteria, most of which are found in the genera *Bacillus* and *Clostridium* can form spores (endospores) when the cells are exposed to unfavorable conditions for growth. Figure 2.11 shows the basic cellular morphologies of bacteria.

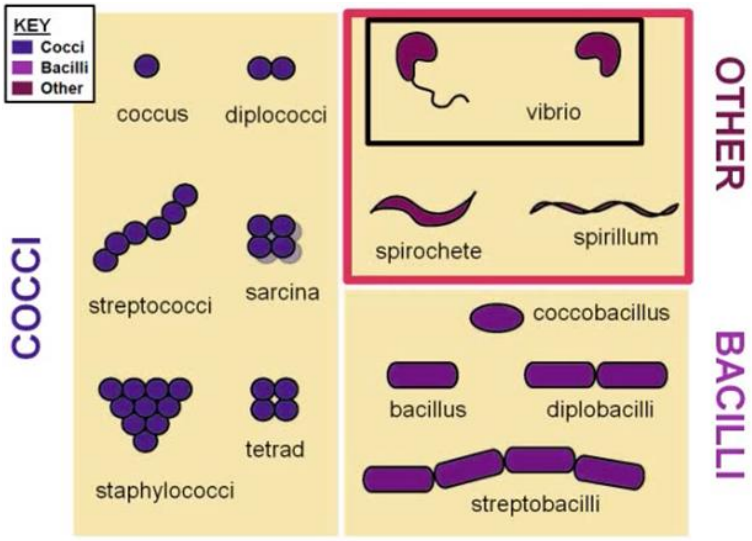


Figure 2.11: Basic cellular morphologies of bacteria
(Source: online, images of Bacteria)

2.8.2 Coliform Bacteria

Coliform bacteria are present in the environment and fecal matter of man and other warm blooded animals. They are characterized as rod shaped, gram negative, non-spore

forming, and motile or non-motile, facultative anaerobic bacteria which can ferment lactose (with the enzyme β-galactosidase) and in the process produces acid and gas when cultured between 36±2°C within 24 – 48 hours (Ashbolt et al., 2001; APHA, 1995). In the context of environment analysis, two categories of coliform bacteria exist: Total coliform and faecal coliform bacteria. Figure 2.12 shows the relationship between total and fecal coliform. Each of them are used as indicator for drinking water quality with each having a different level of risk assessment (Washington State Department of Health, 2011).

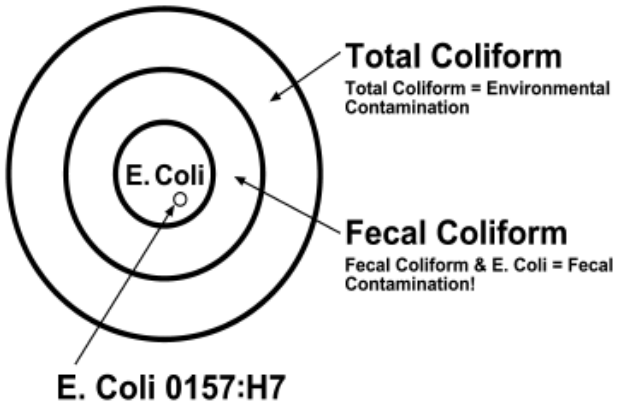


Figure 2.12: Total coliform, Fecal coliform and E. coli relationship
(Source: Online page, coliform Bacteria, 6 February, 2016)

Total coliform bacteria includes all coliform bacteria free living in human, animal and other environmental sources of contamination such as soil and vegetation. Their detection in water does not necessarily indicate pathogen contamination as the majority is nonpathogenic (Washington State Department of Health, 2011). Faecal coliform bacteria, also referred to as faecal indicator organisms, are a subset of total coliform bacteria, and have their origin from faecal matter of human and warm-blooded animals. They are good for predicting the presence of pathogenic bacteria but less good for viruses or protozoan

(Horan, 2003). Two important and common members of this group are *Escherichia coli* (*E. coli*) and the thermo tolerant coliform bacteria (Coliforms that produce acid and gas from lactose at 44.5±0.2°C within 24±2h, also known as faecal coliforms due to their role as faecal indicators) (Ashbolt et al., 2001). *E. coli* is a type of faecal coliform bacteria widely used to predict the presence of pathogenic bacteria in water because it is easy and not expensive to detect, and provides a reliable correlation to the concentration of pathogenic bacteria present in the water sample (World Health Organization, 2003 cited in Foppen & Schijven 2006). Majority of *E. coli* are harmless. However, some specific strains such as *E. coli O157:H7* are capable of causing an outbreak. Examples of pathogenic coliform bacteria of faecal origin include *Salmonella*, which is the major cause of diarrhoea and typhoid fever in immune-compromised adults (Gordon, 2008); *Shigella*, the causer of shigellosis or bacillary dysentery (Cabral, 2010); and *Campylobacter jejuni*, one of the causative agents of gastroenteritis of bacterial origin (Acheson & Allos, 2001). Though not belonging to the faecal coliform bacteria group, the genus *citrobacter* is a member of the group of coliform bacteria commonly found in poor sanitary environment not necessarily of faecal source. They can cause skin, soft tissue, common urinary and lower respiratory tract infections (Fraser et al., 2014; Fraser & Arnett, 2010).

2.8.3 Common bacteria in groundwater

Table 2.1 shows some common bacteria found in groundwater with the diseases they cause and the sources from where they enter into the environment.

Table 2.2: Bacteria found in groundwater
(Source: Krauss & Griebler, 2011)

Bacteria	Major Diseases	Source
Escherichia Coli	Gastroenteritis, Haemolytic Uraemic Syndrome (enterotoxic *E. coli*)	Human faeces
Salmonella spp.	Pneumonia, meningitis, pericarditis, reactive arthritis, enterocolitis, endocarditis	Human and animal faeces
Shigella spp.	Gastroenteritis, bacillary dysentery, reactive arthritis	Human faeces
Campylobacter jejuni	Gastroenteritis, Guillain-Barre Syndrome	Human and animal faeces
Yersinia spp.	Diarrhoea, reactive arthritis	Human and animal faeces
Vibrio cholerae	Cholera	Human faeces and fresh water zooplankton
Legionella spp.	Pneumonia (Legionnaires' disease)	Thermally enriched water
Pseudomonas aeruginosa	Pneumonia, urinary tract infections, bacteremia	Soil and water
Mycobacterium spp.	Pulmonary diseases, skin and soft tissue diseases	Soil and water

2.9 Bacterial detection methods

Technologies for bacterial detection have been gradually become more rapid from the first method known as multiple tube fermentation tests, the traditional methods which take 7-8 days to yield a result, to molecular biological methods. A state of the art technique called biosensor is a promising breakthrough though yet in the development stage (Lazcka et al., 2007). These efforts result from the need for a more reliable and faster method. Three detections types are known for bacteria: quantitative detection (number of bacteria per litre of sample), qualitative detection (presence or absence), and semi-quantitative. Three methods of detection are in practice: Classic detection methods (culture on agar plate and culture on membrane filter), molecular biology methods

(quantitative polymerase chain reaction), and Immunological methods (Dutch National Institute for Public Health, 2014).

The classic/traditional method applies chromogenic compounds added to growth medium. The advent of this method revolutionized bacterial detection by removing the need for isolation of pure cultures and confirmatory tests. These chromogenic compounds are modified either by enzymes (which are typical for the respective bacteria) or by specific bacterial metabolites (Ashbolt et al., 2001). Modification is enhanced when the bacteria are cultured under appropriate conditions (36 ± 2^{o}C for *E. coli* for instance). The chromogenic substance fluoresces or displays colour(s) depictive of the metabolic capacity of the colonies of bacteria present thus enabling easy detection of those colonies. The process generally involve taking a sample, culture or grow them on a petri dish with growth media (agar) such as chromocult for 24 - 48 hours and count the number of colonies formed in cfu ml^{-1} (Colony Forming Units per ml of sample). This method is widely used because it is simple, cheap and reliable.

The membrane filter on plate culture method is a classical method which makes it possible to increase the concentration of bacterial cells for non-pure culture by filtering 100 mL or more of water sample for instance. It is based on entrapment of bacterial cells by a membrane filter (Figueras et al., 2000). There are various types of filters based on the size of the pores. The 0.2 μm cellulose nitrate filters can retain smaller bacterial species than the 0.45 μm cellulose acetate filters. Some filters such as the cellulose nitrate are provided with grid lines that enhances enumeration. The sample water can be forced to pass through the filter membrane using a peristaltic pump or manually operated equipment. The filter is then placed on a ready prepared petri dish with growth media,

and incubated for 24 - 48 hours. The growth of the cells of the bacterial parameter(s) present is manifested by colour appearance, which are then counted and recorded. A popular growth media for bacterial detection is the ChromoCult Agar. It specifically detects *E. coli* and Total coliform with high accuracy standard. Before then, the most probable number method was applied which is based on random dispersion of micro-organisms per volume in a given sample. It has the disadvantage of longer time to acquire result. It however is still in use because of its applicability to virtually all sample types (Figueras et al., 2000).

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

The data collection process involved *field work* and *laboratory work*. The field work entailed:

i. Sanitary inspection survey of the dug wells and boreholes.
ii. Sampling from a total of 22 boreholes and dug wells for virus detection, and 56 for total and faecal coliform bacteria detection.

The laboratory work was done for virus and faecal coliform bacteria detection. For the virus detection, the lab work was done in two stages:

Stage 1: **Up-concentration** of the groundwater samples in a field lab in Dodowa which was established as part of the T-Group. The establishment was coordinated by the Central University College in Accra, Ghana. Viral Sampling and concentration lasted between November and December 2015.

Stage 2: Was **DNA extraction and virus detection** in the molecular biology laboratory at UNESCO-IHE.

After stage 1 of the laboratory work, the concentrated samples were shipped in frozen condition to UNESCO-IHE where laboratory work stage 2 was undertaken by Dr. Jan Willem Foppen, Dr. Jack v d Vossenberg, and Yvonne Hoiting. The materials shown in Appendix 8 were used for each step. The results of laboratory work stage 2 were then shared for analysis and discussion (in chapter four) in line with the research objectives.

Bacterial sampling and culturing were done in January 2016 in the study area and field laboratory.

3.2 Study Area

3.2.1 Location and geology

The study area, Dodowa, is the district capital of the Shai Osudoku District in the Greater Accra Region of Ghana, West Africa. It is a Peri-Urban community located between latitude 5.87 and 5.91^{o}N, and longitude 0.06 and 0.12^{o}W (see figure 3.1). The township is bordered on the north by the Akwapim range that apparently forms the recharge zone of the shallow ground water sources. There are several inland valley swamps on the south and eastern fringes of the township which itself is at a rolling elevation between 1,050 ft to 105 ft above sea level. Figure 3.1 shows the geographic location of Dodowa.

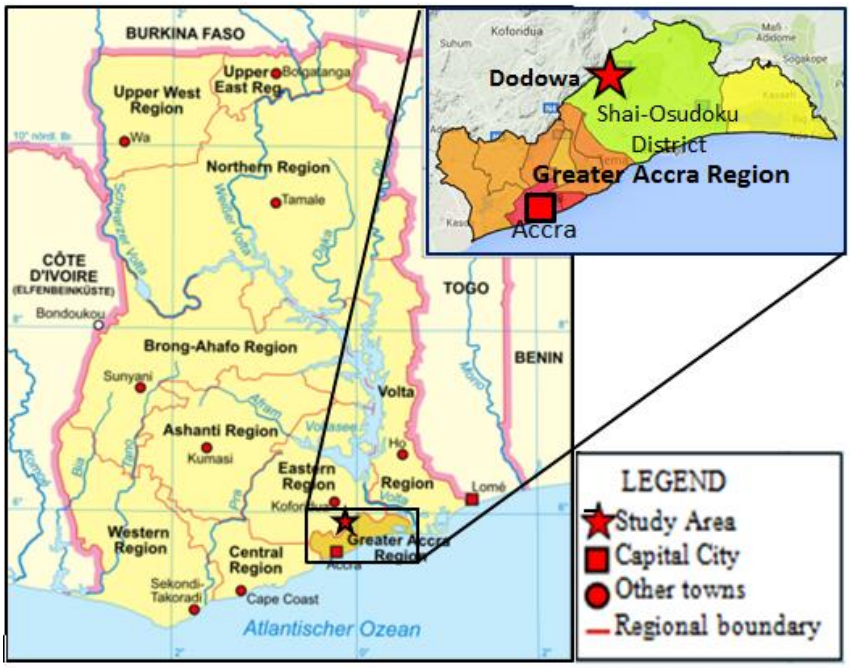


Figure 3.1: Map of Ghana showing the location of study area, Dodowa

(Source: simple.wikipedia.org)

By lithology, the township is underlained by the crystalline basement complex rock formation or quartzite and phyllite belonging to the Togo Structural Unit and hornblende gneiss of the Dahomeyan Structural Unit (Sakyi, 1996; Dampare, 1996; Ayikwei et al., 2011). Groundwater recharge occurs on the Akuaping hills and discharges towards the South-western and South-eastern reaches. A parallel T-group study on the hydro-chemical mapping of groundwater in the study area identified two aquifer types, i.e. the weathered rocks (quartzites and the Dahomeyan gneisses) which extends from the surface to about 20 m (observed from T-Group drilling work in the area – 13^{th}-19^{th} December, 2015), and the deep seated fresh fractured quartzites of the Togo formation and the acidic gneisses. Permeability was found to be high in the fractured aquifer than the weathered. The fractured aquifers were found in the gneisses and have permeability range between 2.3-3 m/day. The weathered aquifers were found in both gneisses and quartzite and with permeability of 0.008 - 0.07 m/d and 0.02 - 0.4 m/d respectively.

3.2.2 Socio-economic Status

Ghana is a middle income nation according to the United Nations human development index ranking 2012, with a per capita income of USD 775.46 per annum as at 2014 (= 2.12 USD/day). The study area portrays a standard lower than national ranking. Despite the availability of public facilities such as schools and health centres, the standard of living as manifested by the physical development of the township at the local end is low. The per capita income was estimated as USD613.20 per annum (i.e. 1.68USD/day)

(Adjei, 2015). Farming and trading are the main economic activities of the majority of the 12,075 inhabitants (census 2010 projection) in Dodowa.

3.2.3 Water resources, supply and usage

A notable and perhaps the only viable surface water body is the Dodowa Stream. It is about 4.5Km long with its source originating from the Akuapim range, northwest of the township. It discharges into a lowland swamp in the south to south-west direction. In the dry season, this stream goes dry. Groundwater is being exploited extensively in the form of dug wells and boreholes. There are over 65 dug wells and about 20 boreholes for private and commercial purpose. In the rainy season, the water table rises to less than a meter below ground level in many areas. High EC values of up to 6000 µS/cm were recorded for some boreholes and dug wells. Though the sources of high EC value could not be determined, the possibility of it partly coming from wastewater infiltration cannot be overruled. However, the chemical content, such as nitrate and phosphate, of groundwater at some sections of the community may need to be investigated because they are nutrients for algae growth observed in some dug wells.

The township is partly supplied with pipe borne water by the national utility, Ghana Water Company at coverage of less than 20%. There are a few private water purification companies that produce sachet and bottled water for commercial purpose. While most residents resort into buying sachet and bottled water for drinking purpose, quite a number cannot afford the cost and depends on borehole sources for both drinking and other domestic purposes. Out of about 300 household interviewed with questionnaires, 38% said that they use groundwater for various purposes including drinking. 93% and 45% of

the users claimed that groundwater in Dodowa is fit for cooking and drinking respectively. 81% of them said that the water is not pretreated before use. Statistics result of household survey done by means of questionnaires, for various forms of water supply means and usage information is provided in Appendix 8 (Adjei, 2015).

3.2.4 Sanitation

In the context of description, Dodowa lacks proper wastewater disposal system. Except for a few roads, 90% of the township lacks a well networked wastewater drainage system. Wastewater is either discharged underground through soak-away or left to run over ground surface to infiltrates into the soil. There are signs that people living close to the stretch of the river dispose of wastewater into the Dodowa stream which runs through densely populated sections of the township. There are a number of pit latrines most of which are shared by 10 to 20 households and a few public toilets for community use. Houses with flush toilet system have their soak-away designed to infiltrate into the ground. There are areas identified as dumpsites but do not appear to be official neither under proper management.

In many areas of Dodowa, sanitation is extremely poor. The effect on groundwater quality is thus imminent. Some hand-dug wells are located as close as 5m from a pit toilet, or soak-away, or an unlined wastewater drain area. The likelihood of wastewater containing pathogen infiltrating into the groundwater system is visible in the areas where they occur. 40% of the hand dug wells are unlined, and 95% of them are generally unprotected. Plates 3.1 shows grey water disposal into the environment in Dodowa.


Plate 3.1: Grey water disposed into the environment in Dodowa

3.3 Water-points sanitary inspection

3.3.1 Objective and procedure

A survey of the environmental sanitation condition and some other possible contamination factor to groundwater in the vicinity of each water-point was undertaken in order to identify their risk level to contamination. The method for sanitary inspection was adopted from Howard et al., 2003. The method from Howard and co-workers adopts a WHO checklist of environmental sanitation factors influencing the risk of contamination of wells. The checklist was adapted to suit site conditions of the study area (see checklist on Appendix 3).

The procedure of the survey involved physical inspection of the wells and their surrounding environment to qualitatively assess their status on 11 risk factors such as level of protection, proximity to septic tank and pit latrines uphill or downhill, etc. (see checklist shown in Appendix 3). The specific diagnostic information (risk factors) that were 'Yes' for the source in question were then summed up to produce a risk score on a scale of 1 to 11. A score of 9-11 indicates very high risk of contamination; 6-8 = high; 3-

5 = intermediate; and 0-2 = low. In the case that a latrine was within 10 m radius and directly uphill of a groundwater source, that source was automatically marked to be under very high risk of contamination. This data was used for the purpose of analysis and discussion of the possible environmental causes or key risk factors influencing contamination of groundwater in the study area. The data was also used to check if there is a relationship between viral and bacterial load, and the sanitary condition of the wells surrounding. The level of correlation will serve as an appraisal for the sanitary inspection method applied.

3.4 Sampling

3.4.1 Sampling points selection

Groundwater-points within the community were mapped using GPS coordinates, to visualize their density and spatial distribution. A total of 62 sampling points (12 boreholes, 46 dug wells and 4 surface water points) were randomly selected within the community. High viral load was expected in the surface water than groundwater. Therefore surface water was sampled expecting that they could serve as positive controls for the concentration and detection protocols. Figure 3.2 below shows the map of sampling points within the community. Appendix 6 provides a list of the coordinates, location and source type of the sampling points. The sources were water points such as shallow and deep dug wells (lined and unlined), and boreholes.

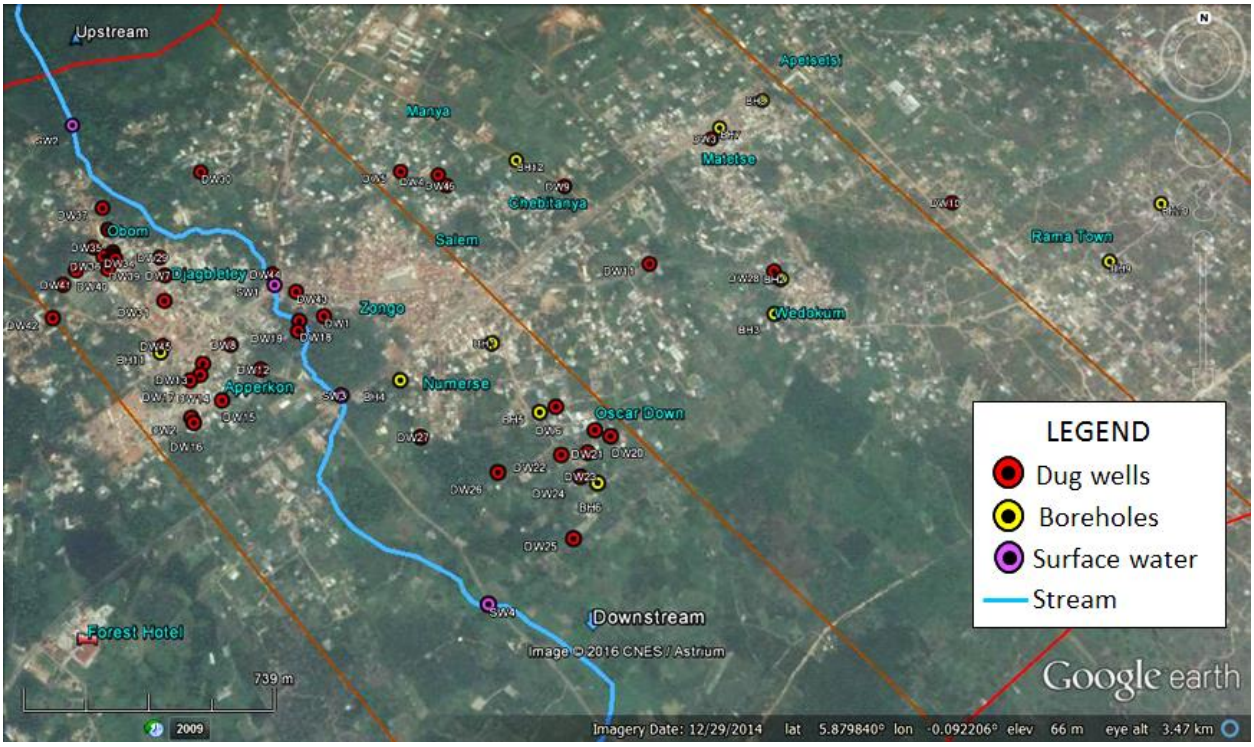


Figure 3.2: Sampling points map

3.4.2 Sample size and quantity

For the viral analysis, 62 water samples were taken from 22 groundwater sources and 1 surface water source at 16 out of the 22 suburbs in the township. For the bacteriological analysis, a total of 56 samples were analyzed from 46 dug wells and 10 boreholes. The virus sampling strategy was based on a spatial and temporal plan in order to attempt assessing the spatial and temporal variation of viral load. Appendix 5 shows the virus sampling scheme developed and applied, and has been summarized below.

Spatial Plan

- 11 dug wells, 11 bore holes and 4 surface water points in 16 out of the 26 suburbs.

- The plan aimed at even distribution within settlements where environmental influence on groundwater is likely. See figure 1 below.

Temporal Plan

- 2 dug wells and 2 bore holes were sampled two or three times a day on a specific week day and repeated after a week. Daily samples were also taken from two dug wells for seven consecutive days. Three out of the four surface water points were resampled after approximately 23 days.

The sample quantity was 100 mL or 20 mL for bacterial test, and 100 L for viral test. For glass-wool filtration protocol, surface water sample has been a minimum of 10 L because it is almost certain that this quantity could contain virus particles in polluted surface water. 100 L was chosen in this research in order to increase the chances of capturing virus particle in groundwater.

3.4.3 Sampling process description

While the principle of accurate sampling such as prevention of cross contamination and proper storage condition cuts across the entire sampling process, the method of sample collection varied with the type of source. Rope and bucket was used to collect sample from dug wells. Hand pump, foot pump or submersible pump was used for bore holes. In general, sampling processes followed the sequence and pattern outlined below.

1. The date and time of sampling was recorded
2. The depth to water was determined with an acoustic depth measuring instrument and recorded.

3. Physical parameters such as pH, electrical conductivity and temperature of the source water were taken on site.
4. Sampling containers were thoroughly rinsed with sample water
5. Samples were collected in 4 of 25 L sample storage containers for virus test, and 250ml sampling bottles for bacterial test and transported to the field lab.
6. In the field lab, the virus test sample was poured in a 100 L container that had been washed with bleach and thoroughly rinsed with tap water the previous day.

3.5 Virus detection and quantitation

3.5.1 Materials

For lab work stage 1, the following materials and reagents were predominantly used:

i. Oiled white glass wool (Insulsafe 12, Asbipro, Schiedam, the Netherlands); 36 grams per column.
ii. Beef extract (3% w/v) in glycine buffer (0.05 M) (as eluant).
iii. Phosphate Buffered Saline (PBS).
iv. Hydrochloric acid (1 N and 0.1 N).
v. Sodium hydroxide (4% w/v (= 1 N)).
vi. Double distilled water.

The reagents were prepared according to the procedure specified in the glass-wool filtration protocol as shown in Appendix 1. Materials for lab work stage 2 are shown in Appendix 8.

The virus detection and quantitation work involved the following processes:

i. Concentrating the groundwater samples,
ii. Extracting virus nucleic acids from the concentrated samples,
iii. Running PCR to detect (and quantify) target viruses (Rotavirus and Adenovirus).

The following sections give a stepwise description of the processes.

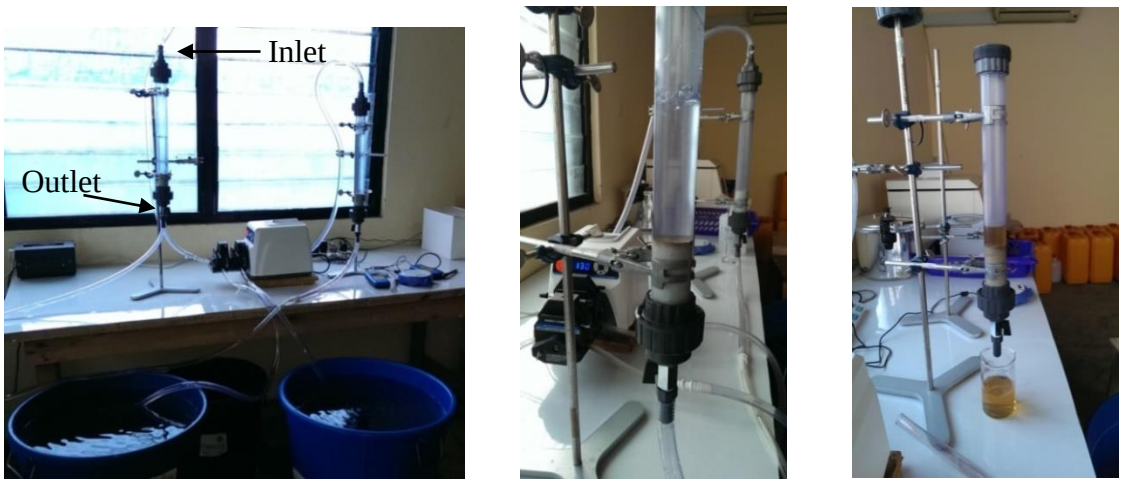
3.5.2 Sample concentration: by glass-wool filtration process

The up-concentration method applied was the glass wool filtration described by Wyn-Jones et al. (2009). Each of the 100 L groundwater samples was concentrated to 10 mL with the aim of retaining most or all virus particles.

The process was done through five steps: filtration, elution, flocculation, centrifugation, and re-suspension. Each of the five process steps are described in the following paragraphs.

Sample preparation and filtration

The 100 L sample water was first conditioned to a pH of 3.5 using 1 N HCl. This is to enable adsorption of virus particles onto glass wool. The 1 N HCl acid was gradually pipetted into the 100 L water sample in a container and stirred continuously until the pH meter reads 3.5. At this state, the water sample has been prepared for filtering through the glass wool filter column. The filtration setup is shown in Plates 3.2.



a) Glass-wool filtration setup b) Filtration process c) Elution process

Plates 3.2: Glass wool filtration setup

36 g of oiled-white (Insulsafe 12, Asbipro, Schiedam, The Netherlands) glass-wool material was packed to a density of about 0.5 g/cm^3 into the transparent plastic column of diameter 40 mm. It occupied an approximate height of 10.5 cm. With the inlet and outlet tubes in place as see in plates 3.2 a), each sample was made to pass through the glass-wool filter column by pumping with a peristaltic pump (Master-flex model) at speed between 120 and 140 rpm. Lab information for each sample concentrated was recorded on a sample up-concentration lab sheet shown in Appendix 2.

Elution

After all the 100 L sample had passed through the glass-wool filter, the glass-wool was eluted (rinsed) with 200 mL of beef extract in glycine buffer solution by pouring the solution into the column through the top-end cap. The eluant whose pH had been raised to 9.5 was poured into the column and allowed to filter through the glass-wool under gravity flow and quiescent condition. The weak electrostatic bond between the virus

particle and the glass-wool material is broken by the high pH eluant. The time for elution varied per sample between 20 and 80 minutes. The eluant was collected in a glass beaker as shown in plate 3.2 c).

Flocculation

The eluted sample which may contain virus particles was then flocculated by lowering the pH with 1 N HCl to the pH of maximum flocculation or turbidity previously determined for the batch of beef extract used. In this research, the pH of maximum flocculation for the batch of beef extract used was 2.82. Beef extract is a protein. Protein surface charges are like many colloidal or sub-colloidal compounds dependent upon pH. When the surface charge is near zero, the proteins tend to be attracted to each other essentially due to van der Waals forces. In this case, the acid lowers the surface charge of all colloidal and sub-colloidal particles as a coagulant.

3.3 a) Beef extract before flocculation (i.e. just after elution)

3.3 b) Beef extract after flocculation

Plates 3.3 a) and b): Eluted sample before and after flocculation

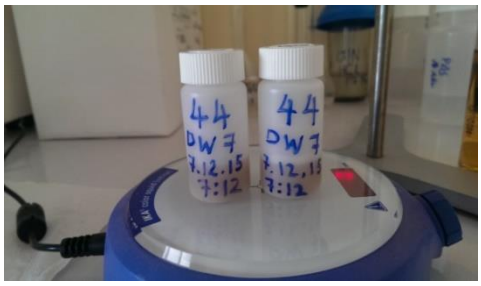
Centrifugation and re-suspension

The flocculated-eluted sample was then poured into centrifuge bottles and centrifuged at 3750 rpm for 30 minutes. Pellets were formed at the bottom of the bottles. The

supernatant was drained off and the pellets were re-suspended with 10 mL Phosphate Buffered Saline using a P5000 pipette. The highly concentrated (approximately 10 mL) sample potentially containing virus particles was then poured into 2 scintillation vials (5ml in each vial) and labeled with source ID, sampling date and time. The PBS preserves the sample from biochemical processes.

Sample storage

After the concentration process, the samples in the vials were kept in a freezer at around -22^{o}C ready to be shipped to UNESCO-IHE molecular biology laboratory in Delft, The Netherlands, where the second stage of lab work was done. If the samples are not frozen, virus particles may break and their nucleic acids inactivated.



3.4 a) 5ml sample in each vial (sample 44 from dug well 7) 3.4 b) Samples in freezer

Plates 3.4 a) and b): Samples in scintillation vials for storage after re-suspension

3.5.3 Nucleic acid extraction

The nucleic acid in virus cells is what is needed to be amplified in the qPCR process and must therefore be extracted from the cells. This stage of the research was done at the molecular biology lab at UNESCO-IHE. The protocol applied was that described by

Boom et al. (1999). The protocol had been checked by Katukiza et al. (2013) for accuracy with sample spiked with bacteriophage PRD1 and *E. coli* DNA and was proven effective.

100 µL sample was taken from the 5mL sample in the scintillation vial to harvest nucleic acids of virus cells that may be present. The principle is that the RNA/DNA is isolated from the sample using a chaotropic lysis buffer and silica colloids or beads to which the nucleic acid binds. After washing, the nucleic acid is eluted from the silica, and can then be used for amplification or sequencing purposes. Detailed description of the protocol is shown in Appendix 4.

Research on groundwater was performed for the first time in such study area. There was no practical clue about the quantity of groundwater sample that can be concentrated to produce enough material. This probably affected the application of the qPCR process for amplification and quantitation of virus genomic copies because the quantity of template nucleic acid may have been below detection level.

3.5.4 Virus detection by PCR

3.5.4.1 Primers and probes synthesis

In the amplification process, primers and probes specific to the nucleic acid to be amplified must be used. For this experiment, primers and probes designed by Hernroth et al. (2002), Allard et al. (2001), and Freeman et al. (2008) were used. They were synthesized by a company called Biolegio, in Netherlands. Table 3.1 shows the primers (forward-F and reverse-R) and probes used.

Table 3.1: Primers and probes used for Adenovirus and Rotavirus amplification

Oligo name and function	Oligo sequence: 5' → 3'	Label 5'	Label 3'	Reference
Adeno-F	CWTACATGCACATCKCSGG	No	No	Henroth *et al.*, 2002
Adeno-R	CRCGGGCRAAYTGCACCAG	No	No	
Adeno-Probe:	CCGGGCTCAGGTACTCCGAGGCGTCCT	6FAM	BHQ1	
Adeno-NVP3-FDeg	ACC ATC TWC ACR TRA CCC TC	No	No	Allard *et al.* 2001
Adeno-NVP3-R	GGT CAC ATA ACG CCC C	No	No	
Adeno-NVP3-R1	GGT CAC ATA ACG CCC CTA TA	No	No	
Adeno-NVP3-Probe	ATG AGC ACA ATA GTT AAA AGC TAA CAC TGT	6FAM	BHQ1	
Rota-NVP3-F	ACCATCTACACATGACCCTC	No	No	Freeman *et al.*, 2008; Pang 2004
Rota-NVP3-R	GGTCACATAACGCCCC	No	No	
Rota-Probe	ATGAGCACAATAGTTAAAAGCTAACACTGTCAA	6FAM	BHQ 1	

3.5.4.2 Rotavirus detection by RT-qPCR

This process was done in the molecular biology lab at UNESCO-IHE, Delft. For the detection and amplification of Rotavirus which is a RNA virus, the RNA was first reverse transcribed into copy-DNA (cDNA) using the RevertAid Reverse Transcription Kit, a two-step system assay manufactured by Thermofisher. Subsequently, qPCR was performed immediately following the reverse transcription reaction. The ingredients with their various quantities mentioned in Appendix 7 were applied and the reaction controlled at each reaction stage according to the manufacturer's instruction for volumes, temperatures, and thermal cycle times as shown in table on Appendix 7. A BioRad MJMini thermal cycler (Miniopticon Real Time PCR system) was used to perform the qPCR assay for the amplification of Rotavirus.

Allard and Freeman primers were applied on target isolates from alternated 250 μL and/or 100 μL concentrated sample volumes. Each sample was tested two, three or four times. The second and third tests were considered as confirmatory tests. The reason was because of the possibility of low concentration of template, and possible presence of inhibitory substances in the sample, a second test for some of the samples would show a contradicting result to the first test. Four batches of tests were thus done in total. A qualitative result (positive, moderate positive or negative) was then produced for all 62 samples. Based on the outcome of the tests, reasonable criteria for decision making were then applied as follows:

Decision criteria:

If at least 2 tests on a sample show positive, that sample was considered positive. If only one out of the two, three, or four tests was positive, the result was regarded as moderate positive. If none of the tests show positive, then that sample is obviously negative.

It is possible that the samples with moderate positive result were as a result of inhibition on the other tests done on them, or is due to low target concentration. However, caution was taken here in deciding because such could not be confirmed immediately and the 1/3 positive result may also have been a false positive. Negative control (NTC) and positive control (RD4) samples were added on each test batch to check for contamination of the experiment.

3.5.4.3 Adenovirus detection by Nested PCR

For the detection and amplification of Adenovirus which is a DNA virus, qPCR assay was first applied with each amplification reaction consisting of Bovin Serum Albumin

solution, dNTP mix, Taq polymerase, Thermopol buffer (10x) mix, forward and reverse primers, and probes (Henroth et al., 2002) shown in Table 3.1. Several runs of qPCR to amplify DNA fragments virtually produced no amplification (suspiciously wrong result) for adenovirus at the expected cycle runs of 37. The Nested PCR assay was then applied to test for positive signals of adenovirus DNA fragments of expected base pairs. This is because of its high sensitivity of amplifying minute fragments just in case it happens that the concentration of DNA fragments in the harvested nucleic acid is below detection level of the standard qPCR process, or inhibition was occurring.

Agarose gel of 1.2% (w/v) (and 2% to visualize small fragments) was used. The DNA samples from all groundwater samples were run under 70 V for 35 minutes. There was a scale or ladder on both sides of the gel lane graduated from 100 – 1000 bp. A minimum band at 171 bp was considered positive for adenovirus (as calculated from its gene sequence). Negative controls (with a non-template sample) and positive controls (with diluted adenovirus sample, AD4) were done for each nested PCR run to check for contamination in the process.

3.5.4.4 Inhibition test

All negative samples of rotavirus and adenovirus tests were tested for inhibition by spiking known concentration (1 μL) of rotavirus or adenovirus to 4 μL isolate sample and the qPCR assay performed according to Freeman, and Heim Protocols for rotavirus and adenovirus respectively. The materials with quantity used in the qPCR mix is shown in Appendix 7.

3.6 Bacteria detection and enumeration

3.6.1 Materials

i. ChromoCult® Coliform Agar (Merck KGaA)

ii. Sterile 0.2 μm cellulose nitrate membrane filters with white grids (Whatman)

iii. Filter holder (Whatman)

iv. Sterile Petri dishes

v. Sterile sampling bottles

vi. Incubator set at 37^{o}C

vii. Ethanol (for disinfection of contaminated surfaces)

3.6.2 Method applied

The culture on plate (with membrane filter) assay with selective growth media (ChromoCult® coliform agar) on petri dish was applied. Cellulose nitrate filter with pores size of size 0.2 μm was used in this study. The advantages for choosing this method were as follows:

i. Since this is a minute filter pore size, it can trap more bacteria cells than other larger size filters (e.g. 0.45 μm).

ii. To increase the chances of accuracy in enumeration of colonies formed due to its grid boxes.

iii. Despite the possibility of finding bacteria cells in the wells, high concentration and even distribution was not too certain. Therefore, by filtering 100 mL of sample, the chances and accuracy of trapping a representative concentration of

bacteria load was expected to increase as compared to plating 100 μL on a petri dish.

The method requires the preparation of agar plates onto which bacteria are cultured. The sample filters were placed in the plate and incubated at specific temperature to enhance growth of bacterial cells. The process description is provided on section 3.6.4.

3.6.3 Preparation of Agar plates

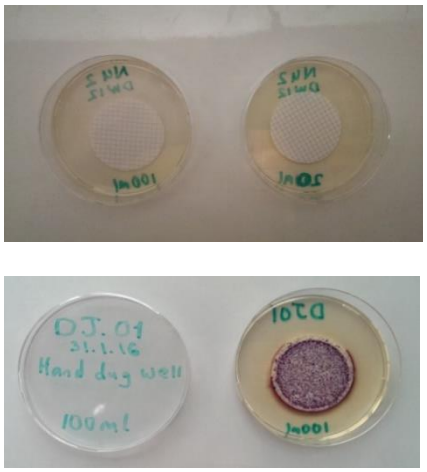
The growth media was prepared by diluting 26.5g of chromocult® agar (Merk) into a litre of demineralized water and heat to 99.9^{O}C in a boiling water bath for a period of one hour. It was left to cool to temperature of 45-50^{O}C and immediately poured on petri dishes about 15 mL per plate. The plates were left to solidify and kept in a refrigerator at 5^{O}C for about 5 days before application of sample-filtered membrane. The chromocult plates were kindly prepared under standard conditions at the microbiology lab of the Central University College, Miotso, Accra, Ghana.

3.6.4 Process description

100 mL of each groundwater sample was filtered in the lab through cellulose nitrate filters. The aim was to determine the number of colony forming units per 100 mL of water sample. It was noticed after culturing the first set of samples that some wells had too many colonies of bacteria to count (i.e. >300 per filter). For this reason, in order to attempt getting countable colonies, for each sample, an extra 20 mL was filtered and plated separately. This means that two culture plates were prepared for each sample. The filters were put on the agar plates. A negative control plate was prepared by pushing

clean autoclaved tap water through a filter as if it was a sample. The plates were then incubated at 37^{O}C for 24 hours. The number of colony forming units per 100 mL of sample water (cfu/100 mL) was counted and recorded. Plates 3.5 a) and b) shows the sample filtration setup and filters in agar plates just before and after incubation.

3.5 a) Sample filtration setup for bacterial test



3.5 b) Sample filters on agar plates just before incubation

Plates 3.5: Sample filtration setup for Bacterial Test

3.6.5 Enumeration and analysis

The chromocult agar product applied specifies the following colour evaluation of colonies:

***Escherichia coli*:** dark blue to violet colonies

***Citrobacter freundii*:** Salmon to red colonies

Total coliform: dark blue to violet colonies, and salmon to red colonies

Other gram negatives: colourless

Two methods of counting were applied. For those colonies that were smaller in number (less than 200), direct manual counting was done. For colonies that were larger in number, an estimated counting aided by the grid boxes of the filter paper was done. Five boxes were selected at random and the average number of colonies of each bacteria parameter in them was calculated. This average number was then multiplied by the total number of effective boxes (approximately 150) of the filter paper. The calculated value is then recorded for the sample for each bacteria parameter in number of coliform unit per 100 mL (cfu/100 mL). In some practical instances, colonies above 300 have been recorded as uncountable or TNTC (Too Numerous To be Counted). For the purpose of further analysis in this research, the estimated counts have been recorded in the result sheet as shown in Appendix 6. The data were analyzed with the aid of MS excel and SPSS data analysis programs. A result of count of one cell and above is regarded as positive for that parameter in that source.

3.7 Analysis of the influence of environmental sanitation to contamination risk

A multi criteria-like analysis was undertaken with two supposedly significant parameters acquired from this study. The aim of the analysis was to arrive at a reasonable assessment of the influence of environmental sanitation factors to groundwater contamination in the study area. These are the key contamination risk factors to groundwater, and the result of the fraction of wells that were vulnerable to each of these factors. The analysis is an attempt to express, in terms of percentage, the level to which environmental sanitation may be influencing pathogen concentration in the dug wells and boreholes in Dodowa. Each risk factor was assumed to have a level of influence (i.e., high, moderate or low). A

value, denoted as *influence factor* (3, 2 or 1, corresponding to high, moderate and low respectively), was assigned to each contamination risk factor based on rational judgment. The product of the *influence factor* and the *fraction of wells* vulnerable to that specific contamination risk factor was computed, and referred to as risk factor *score*. The sum of the scores for the entire environmentally related factors divided by the sum of the scores of all factors (environmental as well as non-environmental) gave the influence of the environment to groundwater contamination in the study area, expressed in percentage (see result tables 4.11 and 4.12).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Sanitary condition and pathogen contamination risk assessment of the wells

4.1.1 General condition of the dug wells and boreholes

Ninety-two percent of the dug wells were either not lined or their lining was not effective thus risking easy infiltration of shallow groundwater and pathogen entry into the wells. All the unlined dug wells had their waters more turbid than the lined wells. Humic materials eroding from the internal surfaces of the weathered rock strata may be responsible. About 95% of the dug wells were not well protected, making it possible for the entry of lizards and other crawling animals, or in the event of droppings from the air (or washout of excreta from animals on top of the well cover). The scooping buckets were highly vulnerable to become infected by this way. It was impossible to inspect the boreholes construction with regards sanitary standards at sub-surface level. At the surface level, six boreholes (BH1, BH5, BH9, BH10, BH11 and BH12) out of the total of 12 inspected did not appear to be properly sealed at the top. Apparently, all six are privately owned and their water is extracted by submersible pump. The remaining six which appeared to be properly sealed at the top were constructed by the government of Ghana and were operated by hand or foot pump. The rubber seals of the foot pump-operated boreholes were all leaking. It was not clear whether or not these pumps were designed to seep water while pumping. The leaking seal could be an entrance for pathogen into the borehole if carried under the foot of the fetcher.

4.1.2 Faecal sludge and wastewater management practices

Inhabitants in the study area practices purely onsite sanitation system. A random survey on toilet facilities indicated that only about 57% of the inhabitants have access to in-house or shared-yard toilet. The rest use public facilities or open defecation on the outskirts of the township. An estimated 72% of the toilet facilities are pit latrines or a less improved system. The 28% that are septic tank are fitted with soak-away pit from where settled wastewater from the septic tanks infiltrate into the ground. There were pit latrines and soak away as close as less than 10 m upstream of some wells as shown in Plate 4.1(a). Grey water from bathrooms was poorly channeled or left to flow into the environment, or to infiltrate into the ground through some local infiltration ditch as shown in Plate 4.1(b).

Plate 4.1(a)

Plate 4.1(b)

Plates 4.1: (a) Pit latrine sited upstream and less than 10m to a dug well, (b) Infiltration/soak-away ditch: common wastewater disposal practice

4.1.3 Contamination risk assessment

To assess the influence of environmental sanitation on the microbial quality of the groundwater in the study area, a sanitary inspection survey was undertaken for all dug wells and boreholes for 11 potential contamination factors resulting from environmental

conditions (see Appendix 3). Based on the number of factors the dug well or borehole was positive for, a risk score of very high, high, intermediate or low was given to each well based on the outcomes of the sanitary inspection result. The objective was to test for a correlation between risk of contamination and the presence and level of contamination found in the wells. Figure 4.1 presents the percentage distribution of contamination risk level from the result of sanitary inspection undertaken for 46 dug wells and 12 boreholes within the study area.

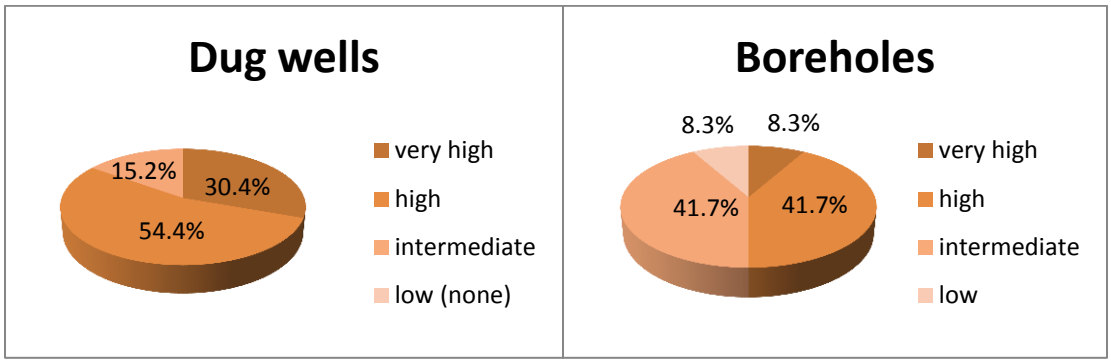


Figure 4.1: percentage distribution of contamination risk level

The results (see figure 4.1 above) indicated that more dug wells were prone to contamination than bore holes. Approximately 80% of the dug wells were at high to very high risk of contamination as against 50% of the boreholes. None of the 56 dug wells inspected was at low risk of contamination, but one out of the 12 boreholes was. 58% (7/12) of the bore holes inspected were constructed for public use by the Government of Ghana under the community water sector. It is apparent that the appropriate procedures for their siting may have to a large extent been followed before they were constructed. However, to what extent those plans have been altered as a result of recent physical developments around these wells so much that contamination risk may have risen, could

not be easily estimated. Research shows that the tendency of upsurge of developments around water points in such settlement is high.

4.1.3.1 Key contamination risk factors to dug wells

Six key contamination risk factors to groundwater in dug wells were identified in this study. For the 46 dug wells inspected, the percentage of wells that were vulnerable to be contaminated by each of these factors was determined and presented in Table 4.1 below.

Table 4.1: vulnerability to contamination of dug wells by key contamination factors

Dug wells, total number: 46

Key Contamination risk factors to dug wells	No. of dug wells	% of total wells
1. Latrine or septic tank soak-away within 10m of the well	22	48%
2. Latrine/septic soak-away at higher ground than well	30	65%
3. Other nearby sources of contamination such as wastewater drain, nearby rubbish dump, animal excreta, etc.	34	74%
4. Rope/bucket left at potentially contaminated point	22	48%
5. Height of apron wall and top protection covering	29	63%
6. Depth and effectiveness of internal lining	28	61%

It could be seen that though about 50% or more of the wells are sited within unsafe proximity to latrines or septic tanks (factors 1 & 2), the majority of wells may not only have been contaminated from these sources but rather from other nearby sources of contamination (i.e. factor 3). From the inspection survey, grey water management was observed to be the single poorest practice in the community. However, this result does not represent the concentration of faecal indicators in the wells, as wells located within short distance from pit latrines as in Plate 4.2b may have higher concentration of faecal

contaminants than those close to other sources of contamination. The fourth key factor is with regards to common practice in handling items used for water extraction in order to separate them from contaminants. The inspection shows that 48% (22/56) of the dug wells inspected have their scooping buckets with rope left around the well for public use. They were assessed to be prone to contamination by animals and other means. It was assumed that those wells which their buckets and ropes are not left in the open (52%) are protected from contamination. The fifth and sixth factors are generally the physical construction of the well. Though a high number of the wells were at risk of contamination by these factors, the consequences of contamination by the first three factors were considered to be more severe.

a): garbage disposed around well

b): well less than 10m downstream of pit latrine

Plates 4.2 a) and b): Wells within unsafe distance from sources of contamination

4.1.3.2 Key contamination risk factors to boreholes

Four key contamination risk factors were identified for the bore holes tested. Table 4.2 shows the number of them that were vulnerable to be contaminated by each of these factors.

Table 4.2: vulnerability to contamination of boreholes by contamination factors

Boreholes, total number: 12

Key Contamination risk factors to boreholes	No. of boreholes	% of total number
1. Unsanitary/worn-out seal	6	50%
2. Nearest latrine a pit latrine that percolates to soil, i.e. unsewered	3	25%
3. Uncapped well within 15-20m of the borehole	1	8%
4. Other environmental source of pollution (e.g. animal excreta, rubbish, and surface water discharge) within 10 m radius	6	50%

4.2 Viral and bacteriological quality results

4.2.1 Rotavirus: RT-qPCR method

None of the 62 samples showed amplification of RNA isolates from the Allard (2001) primers. With the Freeman (2008) primers, there were amplifications in some tests and no amplification in another for the same sample in some instances. Because of its relative responsiveness, the results from the Freeman primers were considered. Based on the decision criteria mentioned in section 3.5.4.2 above, figure 4.2 shows a summary of result for rotavirus tests:

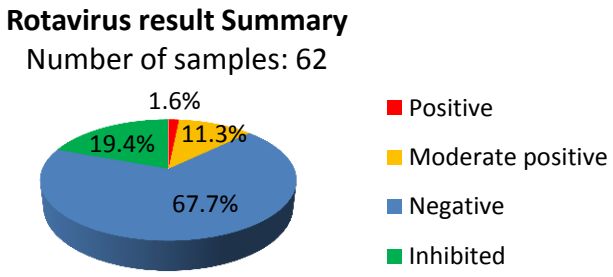


Figure 4.2: Summary of rotavirus result for all 62 samples collected

Only one sample (sample 8, from Dug Well 5, located in Manya Community) was positive for rotavirus. This sample was amplified on two out of three tests done on it.

11.3% (7/62) of the samples showed moderate positive result because only one out of the 3 tests done on each of their samples showed amplification. The two negatives tests may have been inhibited or have got low target concentration. 87.1% (54/62) showed no amplification on all 3 tests and are therefore negative. An inhibition test done for all negative samples indicated that 19.4% (12/62) were inhibited. Table 4.3 gives details of positive, negative, moderate positive and inhibited samples by source type.

Table 4.3: Rotavirus result by source type and sample number

Dug Wells

Well ID	Sample No.	Result
DW1	1	
DW2	3	
DW3	4	
DW4	7	
DW5	8	
DW6	10	
	26	
	27	
	28	
	29	
	34	
	36	
	38	
	40	
	41	
	42	
	59	
DW7	13	
	30	
	31	
	32	
	33	
	35	
	37	
	39	
	43	
	44	
	45	
	60	
DW8	14	
DW9	15	
DW10	21	
DW11	24	
	51	

Boreholes

Well ID	Sample No.	Result
BH1	9	
BH2	11	
BH3	12	
BH4	16	
BH5	17	
BH6	18	
	46	
	47	
	61	
BH7	19	
BH8	20	
	48	
	49	
	62	
BH9	22	
BH10	23	
BH11	25	
	50	

Surface water

Well ID	Sample No.	Result
SW1	2	
	52	
	55	
SW2	5	
	53	
	56	
SW3	6	
	54	
	57	
SW4	58	

LEGEND
Positive
Moderate
Negative
Inhibited

The following tables and qPCR graphs show the results acquired in the laboratory for the four different test batches done.

RT-qPCR Test 1: with Freeman primers, sample 8 to 61 (250µl isolates):

Table 4.4: sample plate setup and amplification cycle of qPCR Test 1

	1 250µl		2 250µl		3 250µl		4 250µl		5 250µl		6 250µl	
	Sample No.	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**
A	8	32.7	26	N/A	37	N/A	55	N/A				
B	9	N/A	28	N/A	38	N/A	56	N/A				
C	14	N/A	30	N/A	39	N/A	57	N/A				
D	16	N/A	31	N/A	40	N/A	58	N/A				
E	17	N/A	32	N/A	50	N/A	60	N/A				
F	18	N/A	33	N/A	52	N/A	61	N/A				
G	19	N/A	34	N/A	53	N/A	**NTC**	**N/A**				
H	25	N/A	35	34.6	54	N/A	**RD4**	**29.3**				

Numbers in red font = amplified samples; C(t) = cycle number; N/A = No Amplification. (Same applies for all four qPCR tests)

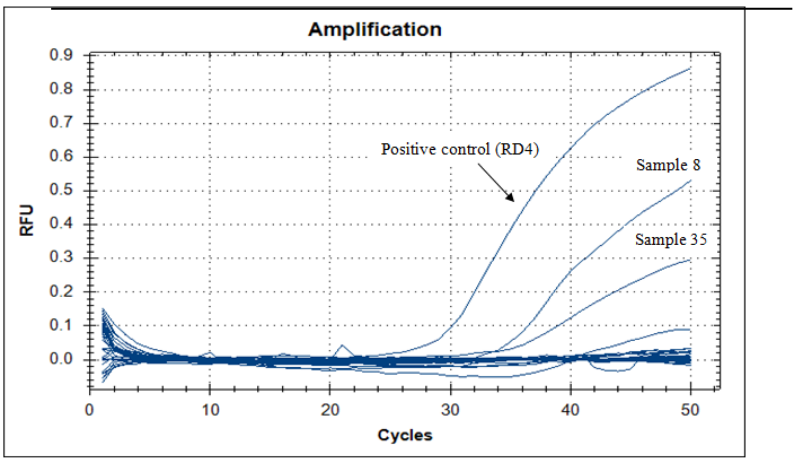


Figure 4.3: Amplification graph of qPCR Test 1

In this test, samples 8 and 35 were amplified after 32.74 and 34.68 cycles respectively, and the positive control RD4 after 29.32 cycles. The NTC showed no amplification indicating that the experiment was not contaminated.

qPCR Test 2: with Freeman primers, sample 1 to 48 (100µl isolates):

Table 4.5: Sample plate setup and amplification cycle of qPCR Test 2

	1 100µl		2 100µl		3 100µl		4 100µl		5 100µl		6 100µl	
	Sample No.	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**
A	1	N/A	9	N/A	17	N/A	25	N/A	33	N/A	41	N/A
B	2	N/A	10	N/A	18	N/A	26	N/A	34	N/A	42	N/A
C	3	N/A	11	N/A	19	N/A	27	N/A	35	N/A	43	N/A
D	4	N/A	12	N/A	20	N/A	28	N/A	36	N/A	44	N/A
E	5	N/A	13	N/A	21	N/A	29	N/A	37	N/A	45	N/A
F	6	N/A	14	N/A	22	N/A	30	N/A	38	N/A	46	N/A
G	7	N/A	15	N/A	23	N/A	31	N/A	39	N/A	**NTC**	**N/A**
H	8	31.1	16	N/A	24	N/A	32	N/A	40	N/A	**RD4**	**28.43**

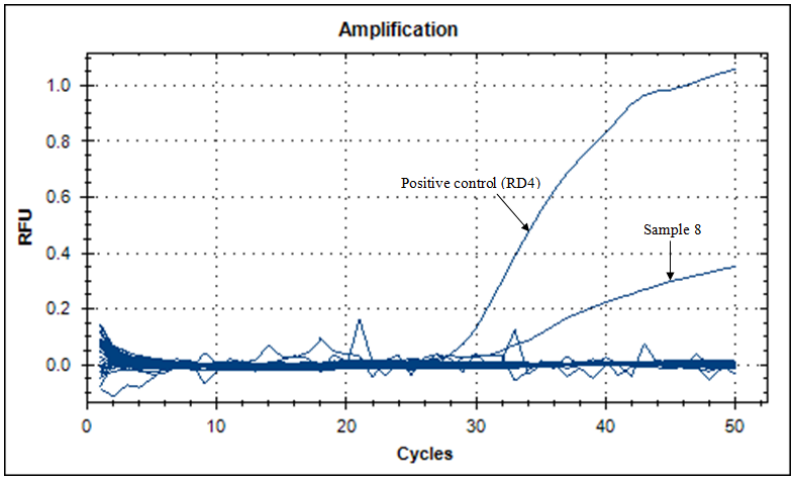


Figure 4.4: Amplification graph of qPCR Test 2

In this test, only sample 8 and the positive control RD4 were amplified after 31.1 and 28.43 cycles respectively. The NTC showed no amplification indicating that the experiment was clean.

qPCR Test 3: with Freeman primers, samples 47-62 (100ul isolate), 1-31 (250ul isolate)

Table 4.6: Sample plate setup and amplification cycle of qPCR Test 3

	1 100µl		2 100µl		3 250µl		4 250µl		5 250µl		6 250µl	
	Sample No.	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**
A	47	N/A	55	N/A	1	N/A	10	N/A	18	N/A	26	N/A
B	48	N/A	56	N/A	2	35.1	11	N/A	19	N/A	27	N/A
C	49	N/A	57	N/A	3	N/A	12	N/A	20	N/A	28	N/A
D	50	N/A	58	36.4	4	N/A	13	N/A	21	N/A	29	N/A
E	51	N/A	59	N/A	5	N/A	14	N/A	22	N/A	30	N/A
F	52	N/A	60	34.69	6	N/A	15	N/A	23	N/A	31	34.1
G	53	N/A	61	N/A	7	31.41	16	N/A	24	N/A	**RD4**	**28.28**
H	54	31.85	62	N/A	8	N/A	17	N/A	25	N/A	**NTC**	**N/A**

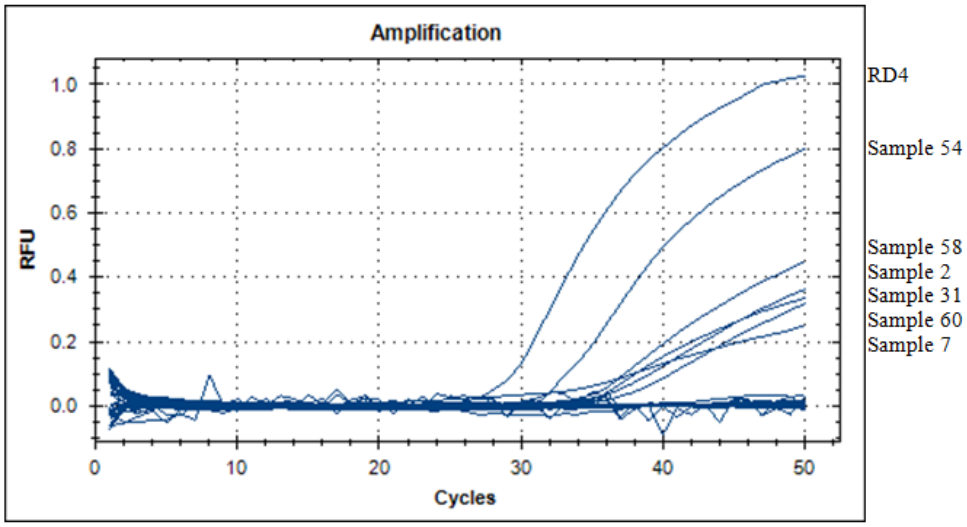


Figure 4.5: Amplification graph of qPCR Test 3

In qPCR test 3, 6 (six) samples; 54, 58, 60, 2, 7, and 31, and the positive control RD4 were amplified. The NTC was not amplified indicating that the experiment was also not contaminated.

qPCR Test 4: with Freeman primers, sample 32-62 (250µl isolate)

Table 4.7: Sample plate setup and amplification cycle of qPCR Test 4

	1 250ul		2 250ul		3 250ul		4 250ul		5 250ul		6	
	Sample No.	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**	**Sample No.**	**C(t)**
A	32	N/A	40	N/A	48	N/A	56	N/A	**RD4**	**29.58**		
B	33	N/A	41	N/A	49	N/A	57	N/A				
C	34	N/A	42	N/A	50	N/A	58	N/A				
D	35	N/A	43	N/A	51	N/A	59	N/A				
E	36	N/A	44	N/A	52	N/A	60	N/A				
F	37	N/A	45	N/A	53	N/A	61	N/A				
G	38	N/A	46	N/A	54	N/A	62	N/A				
H	39	N/A	47	N/A	55	N/A	**NTC**	**N/A**				

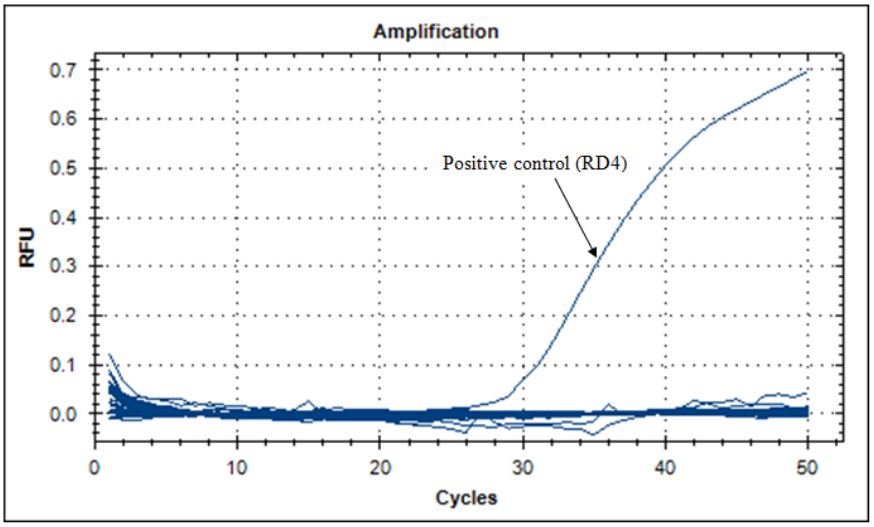


Figure 4.6: Amplification graph of qPCR Test 4

In this test, none of the samples were amplified except the positive control RD4. The NTC was also not amplified indicating no contamination of the experiment.

4.2.1.1 Discussion of rotavirus contamination

Distribution by source type

Figure 4.7 shows the percentage distribution of result for each of the three sources: dug wells (34 samples tested), boreholes (18 samples tested), and surface water (10 samples tested).

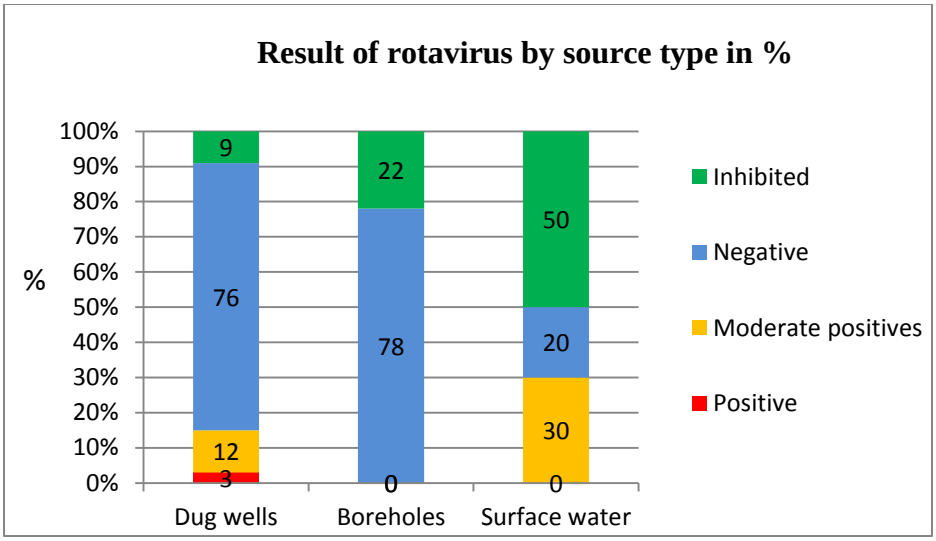


Figure 4.7: percentage distribution of rotavirus results for dug wells, boreholes, and surface water samples

All 18 samples from boreholes were either negative or inhibited for rotavirus. Only one out of 44 samples from dug wells was positive while 4/34 (12%) showed moderate positive result (i.e., these samples were amplified on just one out of 3 tests. A minimum of 2 amplifications on 3 tests was considered as positive). There was no rotavirus in any of the borehole samples while the dug wells had a few positive samples. The boreholes therefore appeared safer than dug wells in terms of rotavirus contamination. None of the surface water samples was positive for rotavirus. This may be surprising because of the higher chances of contamination of surface water than groundwater in such sanitary

environment. However for the purpose of analysis, samples with moderate positive results could be considered as potentially positive. The percentage of moderate positive samples is higher in surface water than dug wells and boreholes. If these samples were to be regarded as positives, the expectation of contamination risk being higher for surface water followed by dug wells and then boreholes is thus supported.

Spatial and temporal variation

Spatially, sample from dug well 5 (DW5) in the Upper Manya Suburb is the only positive sample for rotavirus as shown in figure 4.8. Consequently, the contamination risk assessment rank for this source was 'very high' (8/11). The peculiar risk features of this well were the presence of a septic tank within 15 m, and the absence of good inner wall lining. Figure 4.8 shows the spatial distribution of rotavirus result for positive, moderate, and negative test results.

Figure 4.8: Spatial distribution of rotavirus result of wells/points

Since all borehole samples were negative for rotavirus, there could be no temporal assessment for the two boreholes (BH6 and BH 8) that were sampled for such analysis. For the 2 dug wells (DW6 AND DW7), DW6 has all 12 samples taken at different days and time of the day negative. DW7 also has all 12 samples negative, but with 3 samples taken on different days having moderate positives. Two were samples taken in the morning and the third was taken in the early afternoon. If scooping buckets are carriers of contaminants, it is presumed that ultraviolet rays from the sun may have destroyed the viruses in the afternoon period.

4.2.2 Adenovirus results: qPCR method (Henroth, 2002)

After many thermal cycles with the qPCR assay applied to amplify targets of rotavirus and adenovirus in all 62 samples, questionable amplification occurred. Figure 4.9 shows the resulting curves for 12 samples for Adenovirus detection with no amplification of targets except the positive control, AD3. This was presumed to be as a result of low initial copy number of target nucleic acid. For this reason, the nested PCR method which has higher specificity and sensitivity was applied. The Nested PCR process was able to show amplification for Adenovirus for many samples than the qPCR method did. The amplification process to detect adenovirus in the samples was thus undertaken with the nested PCR method.

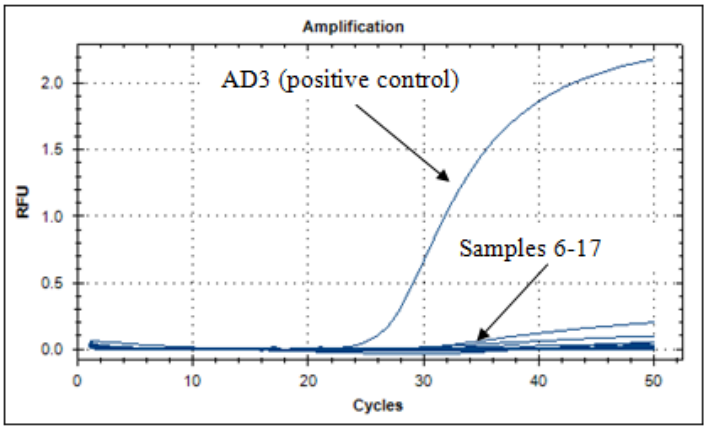


Figure 4.9: Adenovirus amplification result for 12 samples showing no amplification after 30 cycles

4.2.3 Adenovirus result: Nested PCR method

The samples were divided into two batches (1-30 and 31-62), and test ran under the same condition of Agarose gel mix, electromagnetic exposure, and run time. 34 out of the 62 samples were taken from dug wells, 18 from boreholes, and 10 from surface water. Figures 4.10 and 4.11 show the raw laboratory result of the agarose gel runs.

Gel photo and result of samples 1-30: Nested PCR: 1.2% agarose gel, 70V, 35 minutes.

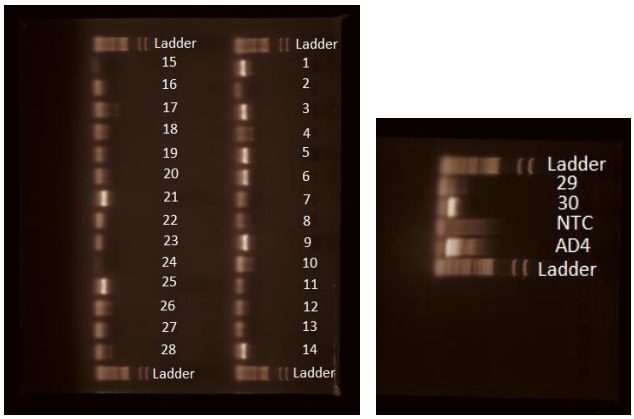


Figure 10: Nested PCR result for Adenovirus for samples 1-30

Samples 1, 3, 5, 6, 9, 14, 21, 25, 30 and the positive control AD4 showed fragments of expected size (171 bp). They are therefore positive for Adenovirus.

Gel photo and result of samples 31-62: Nested PCR: 1.2 % agarose, 70V, 35 minutes.

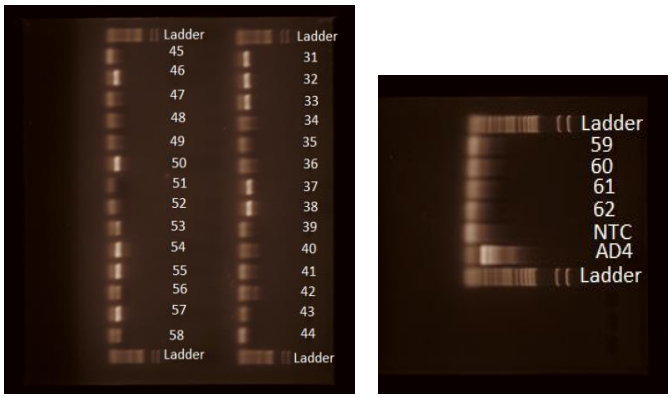


Figure 4.11: Nested PCR result for Adenovirus for samples 31-62

Samples 31, 32, 33, 37, 38, 46, 50, 53, 54, 55, 56, 57, 58 & AD4 showed fragments of expected size (171 bp). These samples were therefore counted as positive for adenovirus. All other samples including the negative control (NTC) only have smaller amplified fragments that can be attributed to primer dimers which means that they were either inhibited or negative for adenovirus. The negative controls of both test batches showed fragments less than the expected implying that they are negative for adenovirus. This confirms that the experiment was free from contamination. Inhibition test done later showed that 12 out of the 62 samples were inhibited. Figure 4.12 shows a summary of result for all 62 samples.

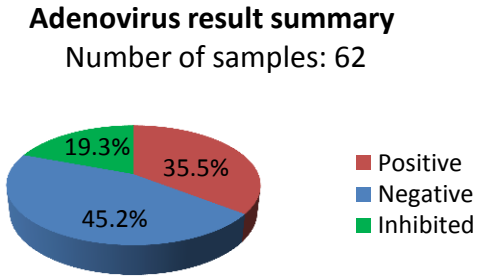


Figure 4.12: Adenovirus test result for all 62 samples

Out of the 22 positive samples, 6 were from boreholes, 10 from dug wells, and the rest were from surface water. Thus, 33% (6/18) of borehole, and 23% (10/44) of dug wells samples were positive.

Table 4.8 gives details of positive, negative and inhibited samples by source type.

Table 4.8:Adenovirus Result by source type and sample number

Dug Wells

Well ID	Sample No.	Result
DW1	1	
DW2	3	
DW3	4	
DW4	7	
DW5	8	
DW6	10	
	26	
	27	
	28	
	29	
	34	
	36	
	38	
	40	
	41	
	42	
	59	
DW7	13	
	30	
	31	
	32	
	33	
	35	
	37	
	39	
	43	
	44	
	45	
	60	
DW8	14	
DW9	15	
DW10	21	
DW11	24	
	51	

Boreholes

Well ID	Sample No.	Result
BH1	9	
BH2	11	
BH3	12	
BH4	16	
BH5	17	
BH6	18	
	46	
	47	
	61	
BH7	19	
BH8	20	
	48	
	49	
	62	
BH9	22	
BH10	23	
BH11	25	
	50	

Surface water

Well ID	Sample No.	Result
SW1	2	
	52	
	55	
SW2	5	
	53	
	56	
SW3	6	
	54	
	57	
SW4	58	

LEGEND

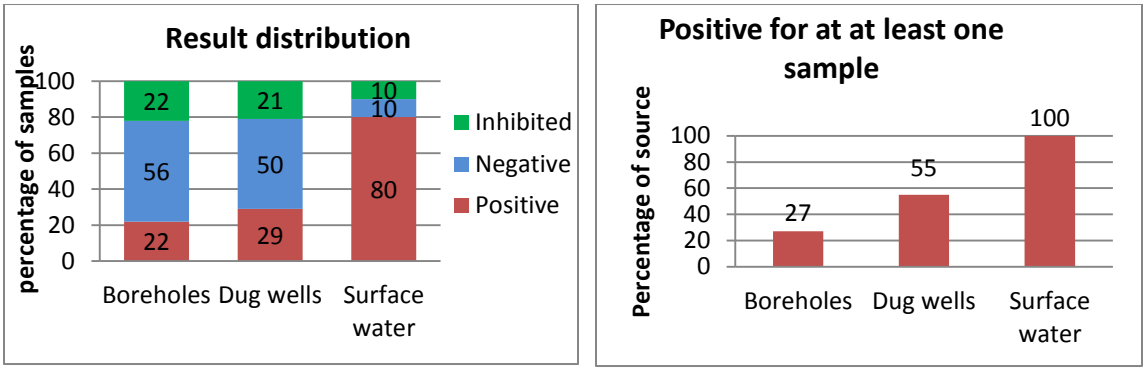
Positive

Negative

Inhibited

Figure 4.13a below presents the percentage distribution of adenovirus result of all 62 samples by source type (34 from dug wells, 18 from boreholes, and 10 from surface water). This is a graphical presentation of table Table 4.8.

Since more than one sample was taken in some wells (DW6, DW7, DW11, BH6, BH8, BH11, SW1, SW2, and SW3), the result for at least one positive for each sampling point or well (11 borehole, 11 dug wells, and 4 surface water sampling points) is shown in figure 4.13 b).



(a) **(b)**

Figure 4.13: Distribution of adenovirus result for dug wells and boreholes, and surface water samples

4.2.3.1 Discussion of adenovirus result

Dug wells vs. boreholes

The fact that more dug wells were contaminated than bore holes may not be a surprise. However, the difference in percentage of positive samples in boreholes and dug wells was not as high as could be expected (figure 4.13a). It was not surprising that almost all surface water samples were contaminated. This could be attributed to the common discharge of wastewater into the stream. About 95% of the dug wells were observed to be in virtually unprotected conditions. Figure 4.13b shows that twice as much dug wells as compared to boreholes had at least one sample positive for adenovirus (i.e. 27% (3/11) of the boreholes as against 55% (6/11) of dug the wells). Despite the low number of

contaminated boreholes as compared to dug wells, it was remarkable to see that boreholes were contaminated even though it was expected that they were constructed under appropriate standards such as with adequate sanitary sealing. But however, no literature ever mentioned the impossibility of such occurrence neither this research presumes or sees the result for bore holes as unexpected in view of the result of high contamination risk to dug wells observed.

Based on the literature on study area description and the result of contamination risk assessment done for each dug well and borehole, an attempt to provide possible conclusion as to why this is so can be better discussed by analysing some of the possible factors such as sources of contamination in the environment, the geology of the area, the properties of these pathogens including their adaptability outside of their host, and perhaps also the groundwater movement profile of the area. These factors may help provide a detailed explanation as to why the balance is shifted to dug wells with regards contamination of adenovirus.

Spatial assessment

Figure 4.14 shows the spatial distribution of adenovirus result for each well sampled. Some wells were sampled more than once (temporal sampling). If at least one sample was positive, that well is reported positive. More positives were seen on the West to South-West of the township as shown in figure 4.14. This area is relatively densely populated than the North to North-East. Quantity of waste production is proportional to population. The proximity of sanitation facilities to groundwater points was also observed to be higher around the densely populated areas like Zongo, Voti, Obom and Djabletey. The above could be reasons why the spatial distribution follows this pattern. As seen

from the spatial map, majority of the adenovirus positives were close to the stream. While it is possible that the stream also influences the presence of pathogen, it could however be hasty to make such conclusion using the available data.

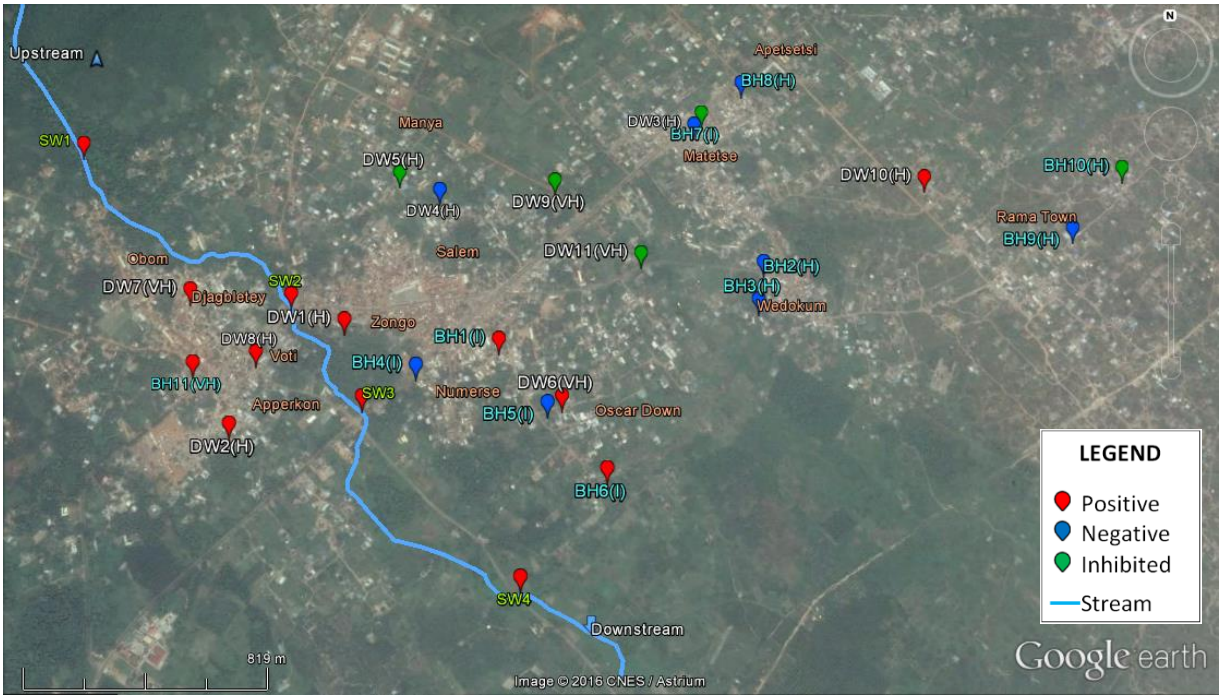


Figure 4.14: Spatial distribution of adenovirus result of wells/points

4.2.3.2 Comparism of rotavirus and adenovirus results on a spatial view

Looking at figures 4.8, and 4.14, a sample from dug well 5 (DW5) in the Manya suburb is positive for both rotavirus and adenovirus, pointing to the fact that there may be a nearby source of viral contamination to this dug well. Three out of the four sampling points along the stream (SW1, SW3, and SW4) were also positive for adenovirus and at the same time had doubtful positives for rotavirus. A peculiar similarity between the two results is that positive or doubtful positive result for adenovirus and rotavirus respectively were taken from wells in close proximity to the stream, or from the stream directly. One difference is that unlike rotavirus, the location of positive samples for adenovirus

appeared to be related to densely populated areas. Another remarkable difference is that some boreholes (BH1, BH6, and BH11) were positive for adenovirus while none was positive for rotavirus. The two viruses have different genomic properties which also is responsible for their different detection and quantitation process (qPCR process for adenovirus, and RT-qPCR for rotavirus). Rotavirus is a RNA virus, and is even more critical to handle in the lab without being degraded by the abundant RNase enzyme in the environment. Thus, the less number of clear positives results observed for rotavirus may be due to the possible influence of its fragile nucleic acid, inhibition, etc.

4.2.4 Bacteriological tests result

Four bacteriological parameters were counted: Total coliform, *E. coli*, *Citrobacter freundii*, and other gram negatives. The focus was *E. coli* which is the faecal contamination indicator bacteria of interest. A result of count of one cell and above is regarded as positive for that parameter in that source. Detailed result of counts of colonies of each bacterial parameter tested for is provided in Appendix 6. Figure 4.15 shows a summary of positive results of the parameters tested per well type in percentage.

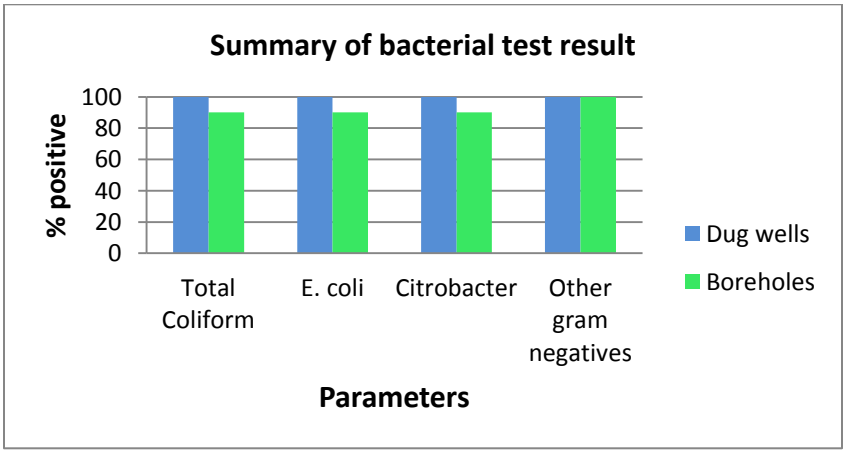


Figure 4.15: Positive results for bacterial parameters tested

All 46 dug wells sampled tested positive for *E. coli*, *Citrobacter and* total coliform. Only one out of the 10 boreholes tested was negative for *E. coli* and citrobacter, and therefore total coliform.

4.2.4.1 Discussion of bacteriological test result

Dug wells

The distribution in CFU counts of the bacterial parameters tested for is presented in figure 4.16.

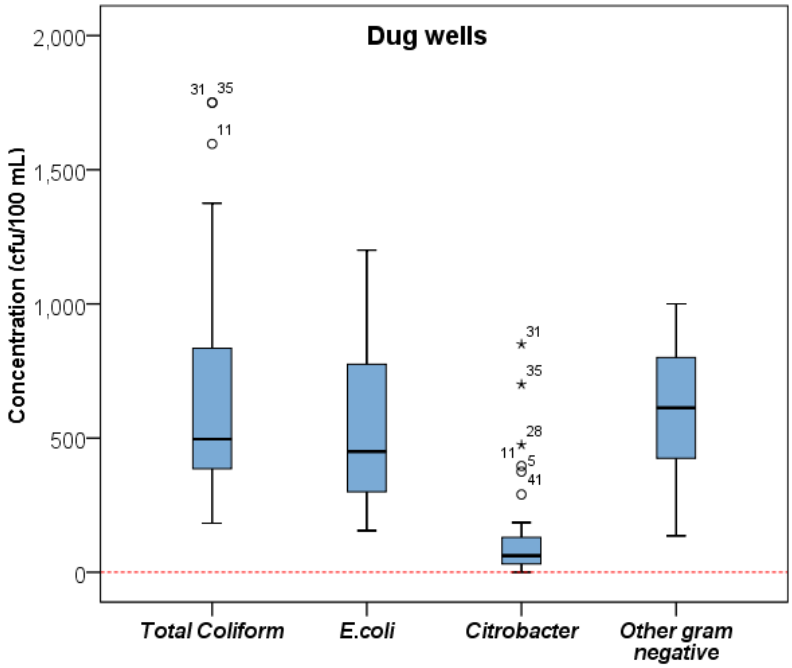


Figure 4.16: Distribution of CFU count for TC, E. coli, Enterobacter, and Salmonella from 46 dug wells

The *E. coli* result in figure 4.16 shows some dug wells with a count of over 1000 cfu/100 mL. At least 75% of the dug wells had *E. coli* count of 250 cfu/100 mL or more. None of the wells had *E. coli* count of zero. Thus they were all contaminated with faecal matter. The least faecal contaminated borehole (DW19) had an *E. coli* count of 155 cfu/100 mL. *Citrorobacter*, though belonging to the coliform group of bacteria, is not a member of the

faecal coliform group. The result shows that a good number of the wells (about 25%) had more than 200 counts. However, there are a few wells (about 4.3%) which were seriously contaminated with over 800 cfu/100 mL. This indicates that within the study area, there could be other sources of infectious bacterial contamination which are not of faecal origin. The sanitary inspection result which indicated that approximately 74% of the dug wells sampled were prone to contamination from sources other than latrines and septic tanks soak-away further supports this result.

Other gram negative bacteria were counted. About 75% of the dug wells contained over 400 cfu/100 mL. The result of *E. coli*, and to some extent the presence of other gram negative bacteria, confirms the presence of pathogenic coliform bacteria thus throwing light to the possible presence of other pathogenic enteric bacteria like *salmonella*, *Shigella*, etc. The red line indicated WHO guideline value. From the result above, none of the dug wells pass the bacteriological quality for drinking water according to the WHO guidelines which stipulates a zero count of *E. coli* for safe drinking water.

Boreholes

Figure 4.17 shows the distribution in CFU counts of Total Coliform and *E. coli* in the 10 bore holes tested.

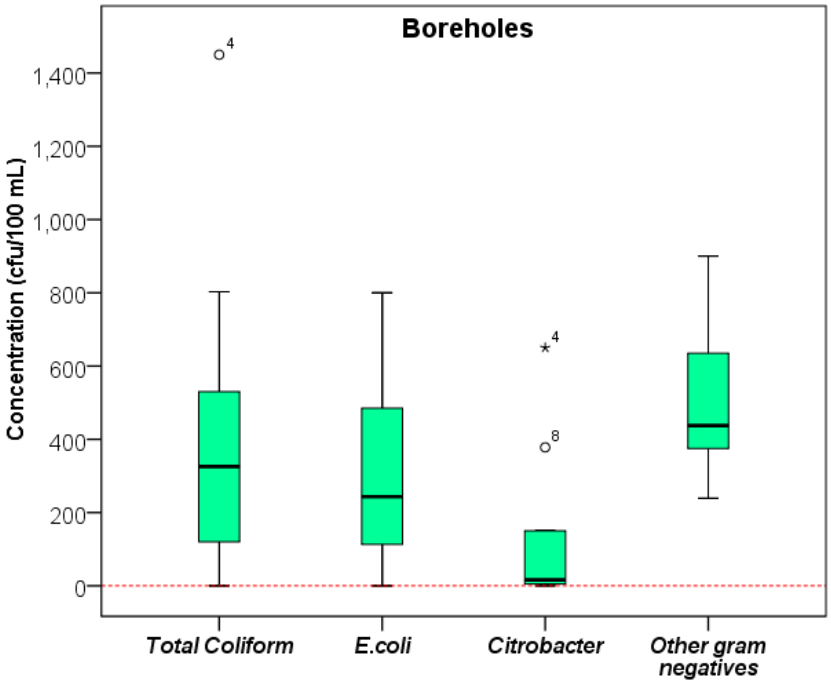


Figure 4.17: Distribution of CFU count for E. coli, enterobacter and salmonella from 10 boreholes

Only one out of the 10 boreholes sampled had zero *E. coli* count. Unlike dug wells, about 50% of the boreholes had *E. coli* count less than 250 cfu/100 mL. *E. coli* positive for 90% of the boreholes indicates that faecal matters may be finding their way into deeper depths of groundwater form the local environment. Three out of the 10 boreholes sampled for bacteria are having their water extracted with a foot pump system. There were signs of leaking valves on the pedal through which pathogen can pass. The borehole that was negative for *E. coli* was operated with a hand pump system.

Boreholes vs. dug wells

Ninety-percent of the boreholes as against 100% of the dug wells had at least one *E. coli* cell in their samples. The distribution also shows that more dug wells had higher counts than boreholes. A larger number (40%) of the bore holes had counts less than 200 cfu/100 mL whereas only 8.7% of dug wells had less than 200 cfu/100 mL. Looking at the higher concentration range also shows a sway to dug wells, having higher bacteria counts than

bore holes. This result supports the expectation that dug wells are more easily contaminated with faecal matter than boreholes. However, the depth of a borehole could influence the presence and concentration of pathogens in them.

Spatial assessment of bacteriological contamination

As could be seen on figure 4.18, the density of wells in Obom, Apperkon, and Oscar Down communities were higher than the other suburbs. But more wells in Obom, followed by Apperkon, had higher concentration of E. coli than Oscar Down despite the fact that Obom is downstream where it could be expeted that pathogens are washed towards. This result indicates that *E. coli* load on groundwater largely depends on the immediate environmental sanitation. During the sanitary survey, more pit latrines were seen on the north-west (Obom and Djabletey area) which constitutes the old settlement area of Dodowa, than Oscar Down where there are many new houses with septic tank system. However the comparative influence of pitlatrine and septic tanks on ground water contamination will be an interesting investigation. But judging from the sanitary information and results that could be assembled in this regard, putting proximity constant, pit latrines appear to have more influence to groundwater contamination particularly in the wet season or if the watertable at any point coincides with the pit, which is something inevitable in many areas of Dodowa due to the high watertable of an average of less than 2m observed during the inspection. Temporal variation of pathogen concentration in the dug wells and bore holes is likely due to seasonal changes. In the rainy season, the water table rises. Faecal contaminants from wastewater withing the community can be quickly transported close to wells apron and infiltration into wells becomes easier. Figure 4.18 shows the spacial distribution of *E. Coli* for various concentration range.

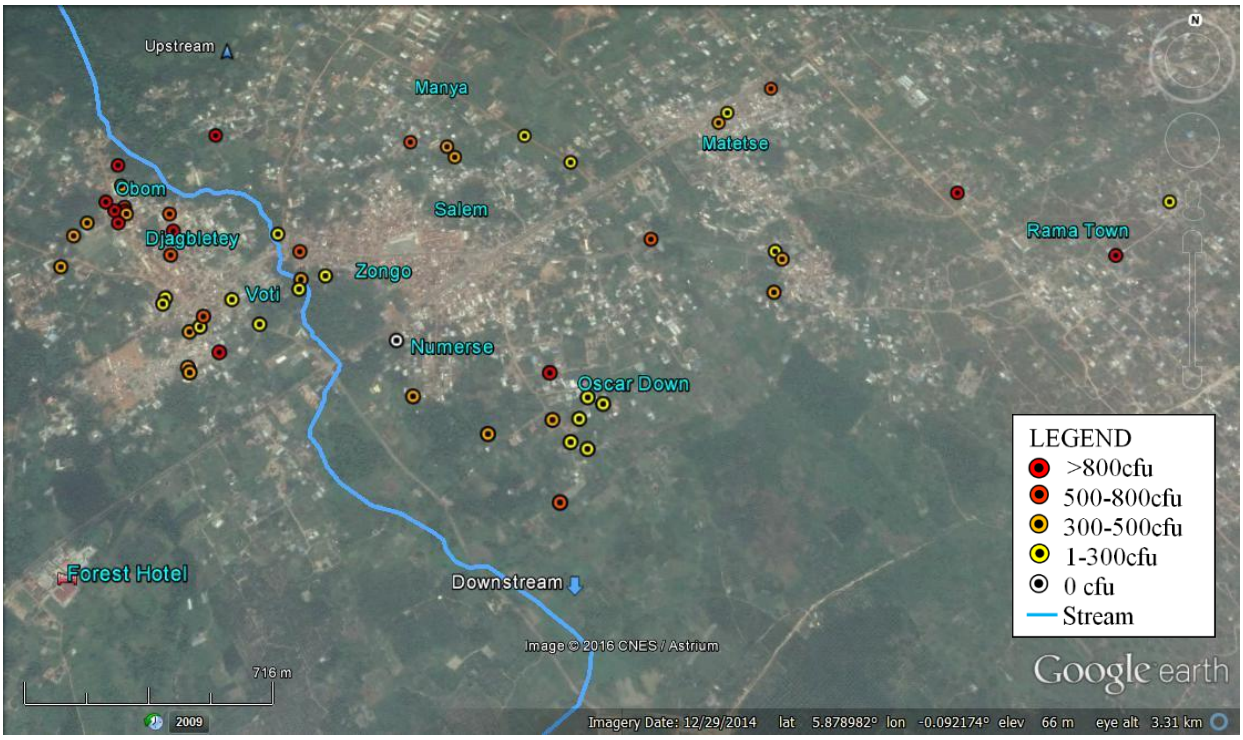


Figure 4.18: Spatial distribution of result of *E. coli* concentration

4.2.4.2 Negative control

Some growth of about 10 and 7 colonies were observed on the negative control plates on two out of three occasions after incubation. But neither of the colonies appeared to be *E. coli*. The colors were dark brown and black. These colors are not part of the colour code of bacterial detection for chromocult agar. The negative control samples were autoclaved for 15 minutes before culturing expecting that all microorganisms have been killed. Cross contamination was doubtful because all necessary precautions were taken to prevent such (The fact that one borehole sample was negative for *E. coli* supports this claim). Also, since the colonies were small in number and were not *E. coli* which is our indicator of interest, they were considered as different bacterial species and thus ignored. However,

the application of chromocult agar for bacterial detection does not guaranteed 100% accuracy for species other than *E. coli*.

4.3 Environmental influence on pathogen quality of groundwater

4.3.1 Environmental sanitation and viral contamination

Tables 4.9 and 4.10 related the viral contamination result to the contamination risk assessment ranking of each well sampled. Sources from which more than one samples were taken was for the purpose of temporal variation assessment.

Table 4.9: Rotavirus result against contamination risk assessment

Source type	Source ID	No. of samples taken	No of samples tested +ve	Result Positive/negative (-/+)	Result of contamination risk assessment study
Bore Holes	BH1	1		-	Intermediate
	BH2	1		-	High
	BH3	1		-	High
	BH4	1		-	Intermediate
	BH5	1		-	Intermediate
	BH6	4		-	Intermediate
	BH7	1		-	Intermediate
	BH8	4		-	High
	BH9	1		-	High
	BH10	1		-	High
	BH11	2		-	Very high
Dug Wells	DW1	1		-	High
	DW2	1		-	High
	DW3	1		-	High
	DW4	1	1	+?	High
	DW5	1	1	+	High
	DW6	12		-	Very high
	DW7	12	1	+?	Very high
	DW8	1		-	High
	DW9	1		-	Very high
	Dw10	1		-	High
	Dw11	2		-	Very high

Table 4.10: Adenovirus result against contamination risk assessment

Source type	Source ID	No. of samples taken	No of samples tested +ve	Result (for at least 1 +ve sample)	Result of contamination risk assessment study
Bore Holes	BH1	1	1	+	Intermediate
	BH2	1		-	High
	BH3	1		-	High
	BH4	1		-	Intermediate
	BH5	1		-	Intermediate
	BH6	4	1	+	Intermediate
	BH7	1		-	Intermediate
	BH8	4		-	High
	BH9	1		-	High
	BH10	1		-	High
	BH11	2	2	+	Very high
Dug Wells	DW1	1	1	+	High
	DW2	1	1	+	High
	DW3	1		-	High
	DW4	1		-	High
	DW5	1		-	High
	DW6	12	1	+	Very high
	DW7	12	5	+	Very high
	DW8	1	1	+	High
	DW9	1		-	Very high
	DW10	1	1	+	High
	DW11	2		-	Very high

In table 4.9, comparing rotavirus result with contamination risk assessment result, the dug wells with positive samples (DW4 and DW7) were at high risk of contamination and DW7 was at very high risk. The four environmental sanitation-related key contamination risk factors shown in Table 4.1 (factors 1 – 4) were common for the dug wells samples that were positive for rotavirus. There was no rotavirus in any of the borehole samples while the dug wells had a few positive samples (Table 4.9). A general reason could be that dug wells are more liable to contamination than boreholes mainly because of their closeness to the surface where there could be higher concentration of faecal contamination. But since rotavirus could not be traced in any of the 18 samples from the 11 boreholes tested, a possibility is that it was absent in the environment, or absent or at an extremely low concentration in the 100 L sample size. It is also likely that some

physical and chemical factors such as straining, temperature, pressure or chemical reaction at deep subsurface level, which could have led to damage of the material forming the capsid, and subsequent deactivation of the virus nucleic acid, may also be responsible. It was seen from the bacteriological test that dug wells were heavily contaminated with *E. coli*. But for Rotavirus, only one out of the 62 samples was clearly positive. The reason could be that there was virtually very low rotavirus contamination of the environment, or absence of it in the majority of sources of fecal contamination like pit latrines and wastewater from the community at the time of inspection.

A similar trend of rotavirus contamination in dug wells in Table 4.9 is also seen for adenovirus in Table 4.10. But unlike rotavirus, there were positive adenovirus samples from boreholes. An interesting outcome of the result of adenovirus in borehole was that two of the three positive boreholes were not at high or very high risk of contamination. This however points to the fact that in addition to immediate environmental sanitation, deep groundwater contamination also depends on other factors such as the properties of the contaminant, the hydrogeology and geochemical characteristics of the area. The sanitary inspection method applied was exclusive of these factors. However, two of the four key contamination risk factors to boreholes were common for BH1 and BH6, and all three were common to BH11, which were the boreholes with positive adenovirus samples.

For viral contamination, the prediction of the sanitary inspection was fairly accurate for the dug wells and less accurate for the bore holes as the result on tables 4.9 and 4.10 indicates. With this, it will not be hasten to conclude that the accuracy of the method of sanitary inspection applied is limited due to other factors not considered in the checklist.

The effect of its limitation likely becomes pronounced in study areas such as Dodowa with a complex geology.

4.3.2 Virus vs. bacteria contamination

The results show that generally, bacterial contamination was high in the community. However, despite the less number of virus positive wells compared to bacteria, the few amplified samples for rotavirus for instance registered C(t) values around 30 (at RFU value around 0.1-0.4) which is not too bad. Practically, C(t) value of say 31 per 4 µL sample is equivalent to about 50 genomic copies (Genomic Copy number/PCR well = $2^{(Ct_{max}-Ct)}$) assuming a Ct_{max} of 37. If this gc value is quantified for 10 mL (10,000 µL) concentrate from 100 L (100,000 mL) sample, we will have: 1.25 gc/mL, i.e.:

$$(50\ gc\ /\ 4\ \mu L) * (10{,}000\ \mu L\ /\ 100{,}000\ mL) = 1.25\ gc\ mL^{-1}$$

This value is equivalent to 125 gc/100 mL and is not too low compared to the range of *E. coli* concentration per 100 mL found in the wells.

Adenovirus however was positive in many (9/22) of the wells tested than rotavirus (which was positive in just 1 out of 22). Just a single well (BH4) was free of all virus and bacterial tests undertaken. The result of many dug wells contaminated with high concentration of *E. coli* (i.e., 75% of them had 300 cfu/100 mL or more) is explained with the fact that bacteria are common in poor sanitary conditions and can easily find their way to the water table where dug wells are normally founded. Also, bacteria are much larger in size and could become trapped in less porous earth materials as they try to make their way through deep groundwater level. Shallow groundwater is thus mostly polluted while deep groundwater is less polluted with pathogens. The above facts are

supported by the results of viral and bacterial load in the samples which shows more dug wells contaminated, and more heavily with *E. Coli* than the boreholes.

4.3.3 Contamination and hydrology: upstream vs. downstream

Groundwater flow is believed to be following the land topography in a subdued form. Judging from this fact and the direction of flow of the Dodowa stream, the direction of groundwater flow under Dodowa is considered from north-west to south-east direction (see figure 4.18). It is believed that because of their immotile nature, virus and bacteria can only travel under subsurface by flowing with groundwater. It was expected that downstream wells could be more contaminated than upstream. However, the spatial distribution maps of rotavirus, adenovirus and *E. coli* (figures 4.8, 4.14 and 4.18 respectively) did not show such conclusive pattern of result between upstream and downstream. The pattern of the spatial result for *E. coli* which showed higher concentrations in wells upstream than downstream further throws light on the fact that the presence of fecal contaminants in groundwater at any point does not only depend on the hydrogeology of the location, but also on how recent the contamination took place, the proximity of the source of contamination to the groundwater point, and the concentration of the contaminant.

The majority of the boreholes sampled (8 out of 12) were located towards the downstream end of the presumed groundwater flow direction (see figure 3.2). Studies on pathogen transport through porous aqueous media show that viruses can travel longer distance and to deeper depth. This could likely be a reason why we saw that two of the

three boreholes contaminated with adenovirus (BH1 and BH6) were located downstream (see figure 4.14).

4.3.4 Environmental sanitation and contamination risk

The sanitary findings on the study area description revealed the predominant use of onsite sanitation system for fecal sludge management, and poor grey water management practices. By coupling these observations and the outcomes of contamination risk assessment, the influence of environmental sanitation to contamination risk could be reasonably assessed.

Eleven contamination risk factors to dug wells and boreholes (Appendix 3) were followed in this study based on the guidelines of Howard et al., 2003. Tables 4.1 and 4.2 present the key contamination risk factors to the wells in the study area and the number of wells that were prone to contamination by each of the key factors. On table 4.1, the first four factors are categorized as environmentally related and the last two are related to the well construction. Tables 4.11 and 4.12 below present a multi-criteria analysis of the influence of those *environmental factors* to contamination of the dug wells and boreholes in the study area. This analysis is an attempt to express, in terms of percentage, the level to which environmental sanitation may be influencing pathogen concentration in the dug wells and boreholes in the study area. Not all the 11 factors, or category of influence factors were considered because it is expected that their level of influence may vary. Thus, three assumptions were made:

1. Based on rational judgement, each risk factor considered has a level of influence of high, moderate or low, corresponding to the values 3, 2 and 1 respectively.

2. The influence of geophysical and chemical factors is constant for all wells sampled.
3. The influence of other factors that were not considered is negligibly small.

Table 4.11: Analysis of environmental influence on pathogen contamination risk to dug wells

DUG WELLS

Key contamination risk factors		*Influence factor (1-3) (A)	Fraction of vulnerable well (B)	Score (A*B)	Total score per category (C)
Category	Risk Factor Description				
Environmental	Latrine or septic tanks soak-away within 10m of the well.	3	0.48	1.44	**5.35**
	Latrine/septic soak-away at higher ground than well.	3	0.65	1.95	
	Other nearby sources of contamination (wastewater drain, nearby rubbish dump, animal excreta, etc.).	2	0.74	1.48	
	Rope/bucket left at potentially contaminated point.	1	0.48	0.48	
Well Construction	Height of apron wall and top protection covering.	1	0.63	0.63	**1.85**
	Depth and effectiveness of internal lining.	2	0.61	1.22	
Total score of all Key contamination risk factors (D)				**7.2**	
Dug wells: Influence of environmental factors to contamination risk ([C/D]*100)					**74%**

*Influence factor: 3 = high, 2 = moderate, 1 = low

Table 4.12: Analysis of environmental influence on pathogen contamination risk to boreholes

BOREHOLES

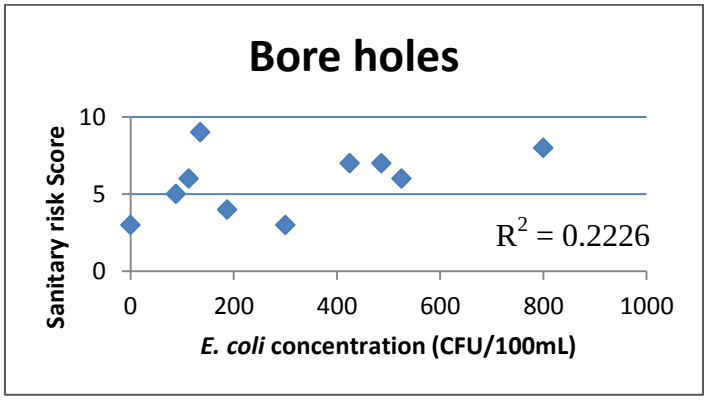
Key contamination risk factors		*Influence factor (1-3) (A)	Fraction of vulnerable well (B)	Score (A*B)	Total score per category (C)
Category	Risk Factor Description				
Environmental	Nearest latrine a pit latrine within 30 m that percolates to soil, i.e. unsewered	2	0.25	0.5	**1.0**
	Other environmental source of pollution (e.g. animal excreta, rubbish, and surface water discharge) within 10 m of the borehole	1	0.5	0.5	
Well Construction	Unsanitary/worn-out seal	3	0.5	1.5	**1.66**
	Uncapped dug well within 15-20m of the borehole	2	0.08	0.16	
Total score of all Key contamination risk factors (D)				**2.66**	
Boreholes: Influence of environmental factors to contamination risk ([C/D]*100)					**38%**

*Influence factor: 3 = high, 2 = moderate, 1 = low

From the analysis in table 4.11, the influence of environmental factors to contamination risk of dug wells in the study area was estimated to be 74%. The remaining 26% is accounted for by the level of physical protection on the construction of the wells. These include the depth and effectiveness of the internal lining, the height of the apron wall and the top covering. The influence of environmental sanitation to contamination of boreholes was comparatively low (38%) (Table 4.12). Unlike dug wells, only two key environmental risk factors could be seen to have significant influence to boreholes. Also, it is believed that the local environment is not very likely to have significant influence on pathogen quality of deep groundwater source as a result of its location and the possibility of the recharge zone being remote from the borehole.

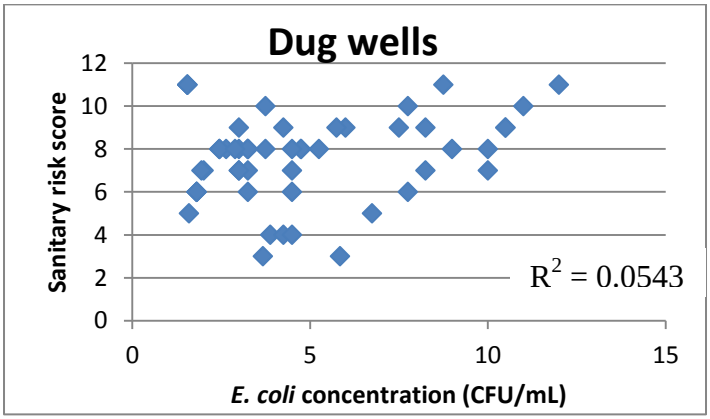
4.3.5 Correlation between environmental sanitation and *E. coli* concentration in wells sampled

Contamination of dug wells and boreholes with faecal matter as depicted by the presence of *E. coli* in these sources may emanate from the immediate environment. But to what extent is the result of sanitary inspection correlates to the concentration of *E. coli* in dug wells for instance is a matter on the grey area. Figures 4.19 and 4.20 below attempts to show the type of correlation that exist between *E. coli* concentration and potential contamination factors in the environment as determined from the sanitary inspection survey.



0 – 12 = No – Very high risk

Figure 4.19: Sanitary risk assessment vs. E. coli concentration in the boreholes



0 – 12 = No – Very high risk

Figure 4.20: sanitary risk assessment score of dug wells against E. coli concentration

The boreholes curve in figure 4.19 shows a weak correlation between the sanitary risk assessment result and the *E. coli* concentration with an R^2 value of 0.223 while the dug well curve (figure 4.20) shows virtually no correlation (R^2 = 0.0543). This outcome is contrary to the conclusion of Mushi et al. (2012) on a similar work in Dar Es Salam, Tanzania where they applied this checklist method. Difference in site condition such as hydrogeology and sanitation may affect consistency of results from place to place. However, after applying a more reasonable analysis with an attempt on data cleaning, it was seen that the *E. coli* result of some of the wells followed the prediction of the sanitary risk assessment result. The analysis is by setting compliance criteria. The criteria are that, a well within a range of *E. coli* concentration from the bacteriological test and at the same time falls within a range of risk score from the contamination risk assessment test result, is considered in the analysis, i.e.,

i. Wells with 0 – 300 cfu and has a contamination risk score between 1 and 5, and wells with >300 cfu and has a contamination risk score between 6 and 11 are considered complying samples.
ii. Non complying samples are assumed to be outliers and therefore ignored.

Considering these criteria, 80% of the boreholes and 61% of the dug wells had their contamination risk score correlated to the *E. coli* load in them. The conclusion this result draws on the applied sanitary inspection method is that the larger the data set, the less accurate the method becomes, and also, other factors such as geophysical and hydro-chemical, that were not considered, may influence the accuracy of the method.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the sanitary inspection undertaken, the following conclusions can be drawn:

I. The dug wells were at high risk of contamination than bore holes. The reason for this was discovered to be due to poor siting of dug wells and/or sanitation facilities like pit latrines, septic tank soak away, and poor grey water management practice.

II. The major risk factor to faecal contamination of groundwater in the study area was poor wastewater management followed by leachate from pit latrines and other environmental sources. In the cases where pit latrines were responsible, there were higher concentrations of indicator bacteria.

III. There was a weak correlation between concentration of *E. coli* in the wells and the result of sanitary inspection. This could mean that there are other parameters that have to be included in the sanitary inspection method for it to accurately predict pathogen concentration. Notwithstanding, the disadvantages of the method applied such as its inability to check sub-surface conditions (on parameters such as hydro-chemical, soil porosity, infiltration rate, groundwater flow pattern, etc.) around a well may have affected the level of accuracy of the result. However, the graph shows some promising outcomes of the method if more factors are included.

The following conclusions are drawn from the bacteriological and viral tests:

I. 100% of the dug wells and 90% of the boreholes in the study area were contaminated with faecal indicator bacteria (*E. coli*) and are therefore unsafe for drinking purpose without adequate disinfection. Poor wastewater management followed by poor siting of sanitation facilities were responsible.

II. In this research, 100 L of groundwater sample was collected for virus detection. Almost all samples showed no amplification with the qPCR assay at expected cycle run but showed amplification after applying nested PCR assay which has a much higher sensitivity. The conclusion drawn here is that the concentration of nucleic acid that could be harvested from groundwater sample to meet minimum detection level for the qPCR assay may depend on the quantity of groundwater sample collected and concentrated. Higher volume of sample water, for example 500-1000 L, may increase the chances of detection.

III. The depth of the boreholes could not be determined neither information on that sought from local authorities. There were unconfirmed reports that some of the boreholes especially private ones were shallow (BH11 for example: 20 m), leaving doubt as to whether their source was actually deep groundwater. In this case, the definition of borehole water will be misrepresented and the result obtained for *E. coli* concentration for some boreholes may have been overestimated due to such misrepresentation.

With regards the environmental risk to pathogen quality of groundwater, the distance from the source of contamination to the well is critical to the presence of pathogens in the source. Some literatures stated a maximum distance of 30m from source of contamination. The chances that *E. coli* cannot be traced without being lysed or strained

between soil particles are likely. In this study, assessment of the possible sources of contamination was not undertaken by practical tests or experiments (such as infiltration rate of leachates, and source tracing) but was rather based on physical inspection and supported by scientific facts and theories from the literature. The guidelines for assessing the risk to groundwater from on-site sanitation gave an insight to the results acquired on the level of contamination (in number of CFU) to the dug wells in particular. However, this method may not very accurately present the reality as judgment was limited to factors surrounding local sanitation conditions only. The likelihood of contamination from a more remote environmental source under the influence of certain invisible hydrogeological condition may limit its accuracy. Notwithstanding, since the focus of this research was on the visible environmental sanitation influence to groundwater contamination, this method provided the required data which after analyzed, pointed to the fact that environmental sanitation has high influence to microbial contamination of dug wells at an estimated level of 74%, and medium to low influence on boreholes at an estimated level of 38%.

5.2 Recommendations

I. Local regulation on site selection of sanitary facilities. This measure is very important in controlling groundwater contamination from human excreta. A minimum offset distance to uphill and downhill of groundwater source could be established. Some studies recommend 20 m uphill and 6 m downhill. However, caution must be exercised in following this recommendation because despite the fact that the hydraulic gradient of shallow groundwater typically follows the ground surface, if the well is equipped with a pump of high extraction rate, the

drawdown rate will be very high such that contaminants could be drawn into the well from downstream.

II. There is need for construction of lined secondary drainages that will receive grey water from tertiary drains (wastewater from bathrooms, kitchens etc.) would be necessary because poor grey water management is a major cause of environmental pollution in the study area.

III. Local regulations on grey water disposal practice which should warrant construction of well protected ducts or tertiary drains from household directly into secondary drains without spillage into the environment.

IV. General sensitization and education on the need to follow local regulations regarding sanitation practices.

V. Over 50% of all the wells in the community are for public use. Some kind of local structure should be put in place for their maintenance and protection against contamination. This however may require funding for which a local scheme can be developed.

VI. Regulation on minimum borehole drilling depth based on geophysical survey report is recommended.

VII. Application of sequencing technique for in-depth investigation of virus sequences present in groundwater will be necessary. With this method, the full genome of all RNA and DNA viruses present in groundwater will be sequenced, and their sources identified within the environment. This data will add value to the quantification of microbial risk on groundwater usage in such communities with poor sanitation.

5.3 Areas of possible future research

I. Groundwater may contain suspended particles which can easily clog the glass-wool filter media as the quantity of sample water passing through the glass-wool filter media increases. The glass-wool filtration protocol should be tested and if necessary redeveloped to handle larger volume of up to 1000 litres of ground water sample. The efficiency of the glass-wool filtration method should be further tested with various quantities of surface water samples (e.g. 10 L, 50 L and 100 L) to understand if there is a correlation between quantity of water sample and concentration of nucleic acid.

II. A model of pathogen transport at sub-surface level which will include the factors influencing it (e.g. physical, biological and if necessary chemical factors) should be developed to validate current theories on risk assessment on groundwater contamination and minimum distance of 30m for siting a groundwater point.

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URL of other online sources utilised

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2. https://www.thermofisher.com/gh/en/home/life-science/pcr/real-time-pcr/qpcr-education/taqman-assays-vs-sybr-green-dye-for-qpcr.html

3. http://unhabitat.org/urban-themes/housing-slum-upgrading/

4. http://www.ghanaiantimes.com.gh/shai-osudoku-records-150-cholera-cases/

APPENDICES

Appendix 1: Chemical preparation and storage

Hydrochloric Acid (1N)

98ml concentrated hydrochloric acid (32%)

1L demi water

Measure 1L of demi water in a measuring cylinder and then pour into a clean 2L glass bottle. Using a 100ml measuring cylinder add 98ml of concentrated hydrochloric acid. Label with the batch number and the expiry date.

	Storage	**Expiry**
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	1 year
In-use	Room temperature	1 year

Hydrochloric Acid (0.1N)

10ml 1N hydrochloric acid

90ml demi water

Measure 90ml of demi water in a measuring cylinder and add to a clean glass bottle. With a 10ml disposable pipette add 10ml of 1N hydrochloric acid. Label with the batch number and the expiry date. For storage and expiry dates see above.

Buffers pH

pH buffers are supplied ready to use and a small volume should be aliquot to a plastic universal when needed for use. After use the aliquot buffer and universal should be discarded.

	Storage	**Expiry**
Supplier's Stock	Room temperature	Supplier's use-by date
In-use	Room temperature	Day of Use

Beef Extract (3%) in Glycine Buffer (0.05M)

60g Beef Extract

7.5g Glycine

2L demi water

Add the beef extract and glycine to a clean 3L conical flask. Add the water and stir with a magnetic stirrer to dissolve. This may take 2 hours. Dispense in 200ml or 400ml volumes. Autoclave to sterilize (example 121 ºC for 15 minutes) store at room temperature

	Storage	**Expiry**
Supplier's Stock	Room temperature	Supplier's use-by date
Stock	Room temperature	4 months
In-use	2-8^{0}C	4 months

Before use the beef extract buffer must be quality controlled. Thereto, place a 10ml portion of autoclaved beef extract in a plastic scintillation vial. Use 0.1N HCl to lower the pH to 3.5 (± 0.1), monitor the pH. Reduce the pH drop by drop until a visible floc forms. The floc must be visible at a pH less than pH 3 for the batch of beef extract to pass QC. Record the result on the beef extract QC Worksheet and use this value for maximum flocculation.

Phosphate Buffered Saline (PBS)

For 1 litre of 1x phosphate-buffered saline (1x PBS buffer) use:

- 8.00 g of NaCl
- 0.20 g of KCl
- 1.44 g of Na_2HPO_4
- 0.24 g of KH_2PO_4
- On a magnetic stirrer, dissolve in 800 ml of MilliQ H_2O
- Adjust the pH between 7.2to 7.4 with HCl or NaOH
- Add milliQ H_2O to volume 1 litter in the volumetric flask.

Sterilize according to local procedures e.g. autoclave at 121°C for 15 minutes. Check the sterility, of each batch made. Label with the volume, batch number and the expiry date.

	Storage	**Expiry**
Supplier's Stock	Room temperature	Supplier's use-by date
Working Stock	Room temperature	4 months
In-use	4^{0}C	4 months
In-media	4^{0}C	1 week

Sodium Hydroxide (1N/4%)

40g sodium hydroxide, 1L demi water

Dissolve the sodium hydroxide in the demi water in a sterile glass beaker. Once dissolved, dispense in 100ml volumes into clean glass bottles. Label with the batch number and expiry date.

	Storage	**Expiry**
Supplier's Stock (solid)	Room temperature	Supplier's use-by date
Stock	Room temperature	4 months
In-use	Room temperature	4 months

Appendix 2: Sample up-concentration lab sheet

Filtration Worksheet *One sheet to be used for every sample*

Method	**GLASS WOOL**	**Sample Number**		**Sample Volume**	
Vessel / Pump		**pH Electrode identifier**			
Starting Temperature		**Starting pH**		**Start Time**	
Filter characteristics		**Conditioning Of Water**		**Filtration**	
Filter weight		pH after Conditioning (pH 3.5 ± 0.1)		Actual volume filtered	
Filter height					
Other		Volume of 1N HCl used		Approximate Time for Filtration	
		Batch Number of 1N HCl			
Elution		**Flocculation**			
Eluant	3% w/v Beef Extract solution	pH after Flocculation: Beef extract (3.0 to 3.5 according to optimum pH value previously determined)			
Batch Number					
Batch Number of NaOH					
pH of beef extract for elution (pH 9.5 ± 0.1)		Batch Number of 1N HCl			
Approximate Time for Elution		Batch Number of 0.1N HCl			
Refrigeration		**Centrifugation**			
Start Time		4000x **g**, 30 minutes			
End Time					
Suspension of Pellet		**Remarks**			
PBS	PBS				
Batch Number					
Date of preparation on the label attached to the reagent		All work done by (Initials)		**Date**	

Appendix 3: Sanitary inspection form for open dug wells and boreholes

I Type of facility OPEN DUG WELL

1. General information: Facility Type: .....
: Town/Suburb: .....
2. Code no.—Address.....
3. Date of visit.....
4. Water sample taken? ..... Sample no. ..... Thermotolerant coliform grade.....

II Specific diagnostic information for assessment Risk

1. Is there a latrine within 10 m of the well?	Y/N
2. Is the nearest latrine on higher ground than the well?	Y/N
3. Is there any other source of pollution (e.g. animal excreta, rubbish) within 10 m of the well?	Y/N
4. Is the drainage poor, causing stagnant water within 2m of the well?	Y/N
5. Is there a faulty drainage channel? Is it broken, permitting ponding?	Y/N
6. Is the wall (parapet) around the well inadequate, allowing surface water to enter the well?	Y/N
7. Is the concrete floor less than 1m wide around the well?	Y/N
8. Are the walls of the well inadequately sealed at any point for 3m below ground?	Y/N
9. Are there any cracks in the concrete floor around the well which could permit water to enter the well?	Y/N
10. Are the rope and bucket left in such a position that they may become contaminated?	Y/N
11. Does the installation require fencing?	Y/N

Total score of risks ..... /11

Contamination risk score: 9–11 = very high; 6–8 = high; 3–5 = intermediate; 0–2 = low

III Results and recommendations

The following important points of risk were noted: ..... (List nos. 1–11) and the authority advised on remedial action.

Signature of sanitarian: .....

I Type of facility DEEP BOREHOLE WITH MECHANICAL PUMP

1. General information: Facility Type: .....
: Town/Suburb: .....
2. Code no.—Address: .....
4. Date of visit: .....
5. Is water sample taken? ..... Sample no. ..... Thermotolerant coliform grade: .....

II Specific diagnostic information for assessment Risk

1. Is there a latrine or sewer within 15–20 m of the pump house?	Y/N
2. Is the nearest latrine a pit latrine that percolates to soil, i.e. unsewered?	Y/N
3. Is the nearest latrine in higher ground?	Y/N
4. Is there any other source of pollution (e.g. animal excreta, rubbish, and surface water) within 10 m of the borehole?	Y/N
5. Is there an uncapped well within 15–20 m of the borehole?	Y/N
6. Is the drainage area around the pump house faulty? Is it broken, permitting ponding and/or leakage to ground?	Y/N
7. Is the fencing around the installation damaged in any way which would permit any unauthorized entry or allow animals access?	Y/N
8. Is the floor of the pump house permeable to water?	Y/N
9. Is the well seal unsanitary?	Y/N
10. Is chlorine not present at the sampling tap?	Y/N
11. Does the installation require fencing?	Y/N

Total score of risks: ..... /10

Contamination risk score: 9–11 very high; 6–8 high; 3–5 intermediate; 0–2 low

III Results and recommendations

The following important points of risk were noted: ..... (List nos. 1–10) and the authority advised on remedial action.

Signature of sanitarian: .....

Appendix 4: Protocol for nucleic acid extraction (30 December 2015)

Purpose

This procedure describes the isolation of RNA and DNA using guanidine thiocyanate and silica colloids according to a SOP procedure from RIVM, based on Boom et. al. (1990).

Principle

The RNA/DNA is isolated from sample using a chaotropic lysis buffer and silica colloids or beads to which the nucleic acid binds. After washing, the nucleic acid is eluted from the silica, and can then be used for amplification purposes.

Fluids required per sample

Per sample:	**Per ca. 500 samples**	Per 1000 samples
500 µl L7	**250 ml L7**	500 ml L7
1000 µl L2	**500 ml L2**	1000 ml L2
1000 µl ethanol 70%	**500 ml ethanol 70%**	1000 ml ethanol 70%
500 µl acetone	**250 ml acetone**	500 ml acetone
35 µl TE buffer	**17.5 ml TE buffer**	35 ml TE buffer
10 µl silica	**5.0 ml silica**	10 ml silica

Materials

500 ml measuring cylinder (height ca. 30 cm, width ca. 5 cm): 1
100 ml pipet: 1
Rubber balloon: 1
100 ml volumetric flask: 3
250 ml volumetric flask: 1
500 ml volumetric flask: 2
250 ml glass beaker: 2
500 ml glass flask: 2
Funnel: 4
pH meter: 1
Water bath (65°C): 1
Magnetic stirrer 1
Eppendorf vessels: many

Consumables

1.5 L milliQ
Silica colloids

50 ml conc. HCl
20 g NaOH
6.05 g Tris
7.44 g Na-EDTA
420 g GuSCN
2.5 ml Triton X-100
250 mg Alpha-casein
From the fridge: Ready-made TE buffer.

Preparation of size-fractionated silica

Suspend 10 g of silicon dioxide, SiO_2 in milliQ water to a total volume of 500 ml in a glass cylinder (height of aqueous column, 27.5 cm; width, 5 cm) and let it settle at unit gravity for 24 h at room temperature.

Dispose of a 480-ml portion of the supernatant by suction, and add milliQ water to a total volume of 500 ml. Re-suspend the silica pellet by vigorous shaking. Let it settle for 5 h at room temperature.

Dispose of a 490 ml portion of the supernatant by suction and add 100 µl of HCl (32%, wt/vol) to adjust the suspension to pH 2. The resulting suspension is sufficient for approximately **1,000 NA purifications**.

Prepare small portions (ca. 10 of 1 ml) in Epps. SC is stable for at least 6 months when stored at room temperature in the dark.

Buffers

Prepare **100 ml 5 M NaOH**. Thereto, dissolve 20 g NaOH in 100 ml milliQ water.

Prepare **500 mL of 0.1 M Tris HCL pH 6.4**. Thereto, dissolve 6.05 gram Tris (mol weight = 121.14 g/mol) in about 400 ml of milliQ water. Bring down the pH to 6.4 with HCl. Add to 500 mL with milliQ water.

Prepare **100 ml of 0.2 M EDTA pH 8.0**. Thereto, dissolve 7.44 gram Na-EDTA (mol weight = 372.2 g/mol) in ca. 75 ml milliQ water, and adjust to pH 8 with 5 M NaOH.

Prepare **ca. 250 ml of buffer L7**. Thereto, dissolve under continuous stirring 120 g of GuSCN in 100 ml of 0.1 M Tris hydrochloride pH 6.4 while heating the solution to 65°C in a waterbath. When completely dissolved, the total volume is around 225 ml. Add 22 ml of a 0.2 M EDTA solution pH 8.0 and 2.6 g of Triton X-100 (=ca. 2.5 ml; density 1.07 g/cm3). Homogenize the solution. Add 1 mg/ml alpha-casein (250 mg).

Prepare **500 ml L2.** Thereto, dissolve under continuous stirring 300 g of GuSCN in 250 ml of 0.1 M Tris hydrochloride, pH 6.4 while heating the solution to 65°C in a water bath. When completely dissolved, the total volume is around 500 ml.

Buffers L7 and L2 are stable for at least 3 months at room temperature in the dark.

Note of caution

Upon contact with acids, GuSCN can produce a toxic gas (HCN). As a precaution, GuSCNcontaining buffers should be prepared in a fume hood. As another precaution, GuSCN-containing waste should be collected in a strong alkaline solution (10 N NaOH, in such an amount that the final concentration could not drop below 0.3 N).

Procedure

Pipette **10 µl of silica** into a 1.5 ml Eppendorf vial, and **add 500 µl L7 buffer**. Add **100 µl sample**. Incubate 30 minutes at room temperature, while shaking .Spin down the pellet for 30 s at 13000 g, and remove the supernatant.

Wash the silica-NA pellet twice with **500µl L2**, twice with **500µl 70% ethanol**, and once with **500µl acetone**. Thereto, suspend the silica pellet by pulse/vortexing the fluid for 3 minutes, centrifuge 30 s at 13,000 g and remove supernatant.

After disposal of the acetone, dry the pellet at 56°C with open lid in an Eppendorf heat block for 10-15 min. Check if the pellet is dry.

Add **55 µl TE buffer**, close the vessel, vortex for 3 minutes, and incubate for 10 min at 56°C. Briefly vortex the vessel again and centrifuge for 2 min at 13,000 x g.

Pipet 50 µl of the supernatant into a new Eppendorf. This fluid containing DNA (and RNA) can be used for further experiments. Store at -70°C.

References

RIVM, 2005. RNA isolatie m.b.v. guanidine thiocyanaat (GTC)/silica gel. SOP nr. MGB/M328 (Copy with JWF).

Boom et. al. (1990). Rapid and Simple Method for Purification of Nucleic Acids, Journal of Clinical Microbiology. 28; 495-503.

Boom et. al (1999) . Improved Silica-Guanidiniumthiocyanate DNA Isolation Procedure Based on Selective Binding of Bovine Alpha-Casein to Silica Particles, Journal of Clinical Microbiology. 37; 615-619.

Appendix 5: Virus Sampling Scheme

Pathogen Transport: Dodowa Groundwater viral Sampling Plan — **Nov-Dec. 2015**

x = 1 sample/day; xx = 2 samples/day; xxx = 3 samples/day

	Well Coordinate		Date																												
			Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	FREE	Mon	Tue	Wed
Sample ID Number	LAT	LONG	17/11	18/11	19/11	20/11	21/11	22/11	23/11	24/11	25/11	26/11	27/11	28/11	29/11	30/11	01/12	02/12	03/12	04/12	05/12	06/12	07/12	08/12	09/12	10/12	11/12	12/12	13/12	14/12	15/12	16/12
Borehole 1	5.8805	-0.0963					X																									
Borehole 2	5.8829	-0.0875						X																								
Borehole 3	5.8817	-0.0877						X																								
Borehole 4	5.8798	-0.0989								X																						
Borehole 5	5.8784	-0.0947								X																						
Borehole 6	5.8762	-0.0929								X														XX							X	
Borehole 7	5.8882	-0.0894									X																					
Borehole 8	5.8892	-0.088									X														xx							x
Borehole 9	5.8838	-0.0771										X																				
Borehole 10	5.8859	-0.0752										X																				
Borehole 11	5.8799	-0.1062											X													X						
Dug well 1	5.8812	-0.1013	X																													
Dug well 2	5.8779	-0.1049		X																												
Dug well 3	5.8877	-0.0896		X																												
Dug well 4	5.8861	-0.0984				X																										
Dug well 5	5.8862	-0.0996				X																										
Dug well 6	5.8786	-0.0943					X							XXX	X		X	X	X	X	XX							X				
Dug well 7	5.8824	-0.1064							X							XXX	X	X	X	X		X	XX							X		
Dug well 8	5.8802	-0.1041							X																							
Dug well 9	5.8858	-0.0944							X																							
Dug well 10	5.8857	-0.0819										X																				
Dug well 11	5.8833	-0.0916											X													X						
surface water (US)	5.8822	-0.103	X																								XX					
surface water (MS)	5.8875	-0.1102			X																						XX					
surface water (DS)	5.8787	-0.1006			X																						XX					
surface water (fDS)	5.8728	-0.0959																									X					

Appendix 6: Sampling points information and results of counts of colonies of bacteria cells for boreholes and dug wells

No.	Lat.	Long.	Location	Well ID	TCC /100mL	*E.coli* /100mL	Citro-bacter	Other gram-negative	Contamination risk assessment result
1	5.87946	-0.103086	Aperkon	DW12	242	195	47	425	7(high)
2	5.880212	-0.104092	Aperkon	DW8	226	201	25	445	7(high)
3	5.879589	-0.104825	Aperkon	DW13	835	675	160	800	5(intermediate)
4	5.879241	-0.104851	Aperkon	DW14	327	264	63	875	8 (high)
5	5.878467	-0.104093	Aperkon	DW15	1375	1000	375	900	7(high)
6	5.877860	-0.104885	Aperkon	DW16	482	375	107	375	10 (very high)
7	5.877917	-0.104910	Aperkon	DW2	564	475	89	800	8(high)
8	5.879059	-0.105128	Aperkon	DW17	408	388	20	800	4(intermediate)
9	5.881029	-0.102129	Votti	DW18	411	325	86	375	6(high)
10	5.880700	-0.102151	Votti	DW19	183	155	28	136	11(very high)
11	5.878567	-0.094222	Numerse	DW6	1596	1200	396	800	11(very high)
12	5.877722	-0.092606	Numerse	DW20	386	245	141	775	8(high)
13	5.877886	-0.093070	Numerse	DW21	260	181	79	800	6(high)
14	5.877113	-0.094028	Numerse	DW22	325	325	0	725	8(high)
15	5.877207	-0.093258	Numerse	DW23	199	160	39	350	5(intermediate)
16	5.876485	-0.093442	Numerse	DW24	404	289	115	475	8(high)
17	5.874689	-0.093576	Numerse	DW25	645	585	60	900	3(intermediate)
18	5.876539	-0.095837	Numerse	DW26	399	368	31	475	3(intermediate)
19	5.877525	-0.098160	Numerse	DW27	510	425	85	950	9(very high)
20	5.88572	-0.08193	Rama Town	DW10	837	825	12	675	7(high)
21	5.88774	-0.08963	Matetsi	DW3	521	475	46	475	8(high)
22	5.88326	-0.09156	Wedwkum	DW11	811	750	61	675	9(very high)
23	5.88314	-0.08769	Wedwkum	DW28	343	300	43	450	7(high)
24	5.88239	-0.1064	Djabletey	DW7	1145	1100	45	375	10(very high)
25	5.88298	-0.10666	Djabletey	DW29	823	775	48	775	10(very high)
26	5.88594	-0.10584	Djabletey	DW30	1155	1000	155	800	8(high)
27	5.88156	-0.10629	Djabletey	DW31	905	775	130	325	6(high)
28	5.88298	-0.10808	Obom	DW32	1300	825	475	550	9(very high)
29	5.88311	-0.10812	Obom	DW33	655	600	55	475	9(very high)
30	5.88296	-0.10839	Obom	DW34	1060	875	185	875	11(very high)
31	5.88324	-0.10874	Obom	DW35	1750	900	850	975	8(high)
32	5.88288	-0.108	Obom	DW36	453	425	28	425	4(intermediate)
33	5.88459	-0.1087	Obom	DW37	1117	1050	67	875	9(very high)
34	5.88386	-0.10841	Obom	DW38	466	450	16	875	8(high)
35	5.88254	-0.10817	Obom	DW39	1750	1050	700	1000	9(very high)
36	5.88246	-0.10913	Obom	DW40	422	325	97	450	7(high)
37	5.88198	-0.10943	Obom	DW41	464	450	14	725	6(high)

37	5.88089	-0.10953	Obom	DW42	467	450	17	725	7(high)
39	5.8812	-0.1014	Zongo	DW1	318	300	18	375	8(high)
40	5.88197	-0.10234	Zongo	DW43	715	575	140	825	9(very high)
41	5.88253	-0.10314	Zongo	DW44	590	300	290	418	7(high)
42	5.88011	-0.10611	Lower	DW45	246	225	21	375	8(high)
43	5.88584	-0.09431	Chabitanya	DW9	332	300	32	300	9(very high)
44	5.88617	-0.09953	Manya	DW5	588	525	63	525	8(high)
45	5.88609	-0.09833	Manya	DW4	409	375	34	375	8(high)
46	5.88572	-0.09803	Salem	DW46	540	450	90	475	4(intermediate)
47	5.876314	-0.092940	Numerse	BH6	201	187	14	239	4(intermediate)
48	5.879241	-0.098909	Numerse	BH4	0	0	0	425	3(intermediate)
49	5.8859	-0.07521	Rama Town	BH10	120	113	7	254	6(high)
50	5.88381	-0.07713	Rama Town	BH9	1450	800	650	900	8(high)
51	5.88922	-0.08795	Apartetsi	BH8	530	525	5	675	6(high)
52	5.88815	-0.08936	Matetsi	BH7	91	88	3	450	5(intermediate)
53	5.88288	-0.08747	Wedwkum	BH2	503	485	18	635	7(high)
54	5.88172	-0.0877	Wedwkum	BH3	803	425	378	530	2(low)
55	5.87991	-0.10614	Lower	BH11	172	135	37	378	9(very high)
56	5.88671	-0.09589	Manya	BH12	450	300	150	375	3(intermediate)
57	5.88046	-0.09626	Manya	BH1	NS	NS	NS	NS	5(intermediate)
58	5.87838	-0.09472	Manya	BH5	NS	NS	NS	NS	4(intermediate)
59	5.88218	-0.103025	Dodowa	SW1	NS	NS	NS	NS	
60	5.88745	-0.11015	Dodowa	SW2	NS	NS	NS	NS	
61	5.87873	-0.10061	Dodowa	SW3	NS	NS	NS	NS	
62	5.8728	-0.09587	Dodowa	SW4	NS	NS	NS	NS	

Key: Yellow highlight are the boreholes (BH = Bore Holes, DW = Dug Wells); Red font colour are sources sampled for virus; NS means Not Sampled for bacteria; Counts over 200 were estimated using the filter grid boxes.

Appendix 7: Materials used in RT-qPCR

Step	Reagent	Volume per reaction
1. Nucleic acid extraction (See Boom protocol):	L7	500µl
	L2	1000 µl
	Ethanol 70%	1000 µl
	Acetone	500 µl
	TE buffer	35 µl
	Silica	10 µl
2. Nested PCR (for Adenovirus)	Sample or template	4 µl
	10X Sigma buffer	2.5 µl
	dNTP mix	1 µl
	Taq polymerase (Genscript)	1 µl
	DEPC treated water	14 µl
	Forward primer (Biolegio)	1.25 µl
	Reverse primer (Biolegio)	1.25 µl
	Random primers	1.25 µl
3. RT-qPCR (for Rotavirus):		
3.1 Denaturation and annealing:	70 °C for 5 min	
	Sample or template	4 µl
	Random hexamer (Fermentas)	0.3 µl
	DEPC treated water	8.7 µl
3.2 Reverse transcription:	25 °C for 10 min, 42 °C for 60 min, 70 °C for 10 min	
	RevertAid reverse transcriptase (60 units)	0.3 µl
	dNTP mix (4 mM of each dNTP)	1.25 µl
	5X RT buffer	5 µl
	DEPC treated water	4.45 µl
	Total	**25 µl**
3.3 qPCR	95 °C for 5 min, 40 x (94 °C for 20 s, 60 °C for 60 s)	
	Sample or template	4 µl
	10X Thermopol buffer	2.5 µl
	Bovine Serum Albumin solution (20 mg/ml = 2%)	0.25 µl
	dNTP mix	1 µl
	Taq polymerase (Genscript)	1 µl
	DEPC treated water	15.15 µl
	Forward primer (Biolegio)	0.4 µl

	Reverse primer (Biolegio)	0.4 µl
	Probe (Biolegio)	0.3 µl
	Total	**25 µl**
4. Inhibition Test for Adenovirus (Heim Protocol)	**Rotavirus; adenovirus**	
	Sample or template	4 µl
	Positive (RD6; AD6)	1 µl
	10X Thermopool buffer; 10XSigma buffer	0.25; 2 µl
	Bovin Serum Albumin solution (20mg/ml=2%); MgCl2	0.25;1.6 µl
	dNTP mix	1 µl
	Taq polymerase (Genscript)	1 µl
	DEPC treated water	14.15; 6.6 µl
	Forward primer (Biolegio)	0.4; 1 µl
	Reverse primer (Biolegio)	0.3; 1 µl
	Probe (Biolegio)	0.3; 0.8 µl
	Total	**25; 20 µl**

Appendix 8: Statistical survey result of water supply sources and usage

DRINKING		Fit for Drinking		Total
		Yes	No	
B1.1 Source	Public Tap/Stand Pipe	106	5	111
	Piped into building(indoor/yard tap)	58	4	62
	Borehole (Hand pump/Foot pump)	7	5	12
	Borehole (with Motorized Pump)	14	3	17
	Protected dug well	16	23	39
	Unprotected dug well	14	31	45
	Tanker/Vendor water/cart	0	3	3
	Poly tank or Similar	5	0	5
	Others	5	1	6
Total		**225**	**75**	**300**

COOKING		Fit for cooking		Total
		Yes	No	
B1.1 Source	Public Tap/Stand Pipe	111	0	111
	Piped into building(indoor/yard tap)	62	0	62
	Borehole (Hand pump/Foot pump)	12	0	12
	Borehole (with Motorized Pump)	16	1	17
	Protected dug well	36	3	39
	Unprotected dug well	41	4	45
	Tanker/Vendor water/cart	3	0	3
	Poly tank or Similar	5	0	5
	Others	6	0	6
Total		**292**	**8**	**300**

TREATMENT		Source pre-treatment		Total
		Yes	No	
B1.1 Source	Public Tap/Stand Pipe	101	9	110
	Piped into building(indoor/yard tap)	62	0	62
	Borehole (Hand pump/Foot pump)	3	9	12
	Borehole (with Motorized Pump)	14	3	17
	Protected dug well	4	35	39
	Unprotected dug well	0	45	45
	Tanker/Vendor water/cart	1	2	3
	Poly tank or Similar	0	3	3
	Others	2	4	6
Total		**187**	**110**	**297**

Appendix 9: Photos of sampling and field lab work