# PREVALENCE AND ANTIBIOTIC RESISTANCE PATTERNS OF *Aeromonas* SPECIES FROM DRINKING WATER IN RURAL HOUSEHOLDS' CONTAINERS IN VHEMBE DISTRICT OF SOUTH AFRICA.

by

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A DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS OF THE MASTERS DEGREE IN MICROBIOLOGY (MSCMB)

to the

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

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April 2017

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# DEDICATION

I dedicate this thesis to my family for their endless love and support that encourage me to complete this thesis.



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# ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to the following persons and institutions for their kind contributions and support that was so helpful to the completion of this study:

Prof Potgieter N for her patient supervision, scientific guidance and time during the course of the study;

Prof Traore AN for all her valuable support, motivation and assistance for the progress and completion of this thesis;

Prof Bezuidenhout C and the University of North West Microbiology Department for allowing me to perform molecular section of this study in their laboratory, his scientific input and time;

The University of Venda microbiology department staff members and all my postgraduates' colleagues for their assistance and encouragement;

The National Research Foundation award and University of Venda for their financial support;

The Dzingahe and Ngudza communities for allowing me to collect water samples from their households' containers;

Rohan Fourie and Lesego, for all their assistance and time with the PCR work of the study;

My parents and all family members for their inspiration and financial support.



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# DECLARATION

I, Swalivha Khumbudzo (Student no: 11582681), declare that this dissertation for the Master of science Degree in Microbiology (MScMBY) submitted at the University of Venda is my own work and has not previously been submitted for a degree at any other institution and that all the references materials contained herein have been duly acknowledged.

Swalivha ..... 2017-08-29 .....

Signature date



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# LIST OF ABBREVIATIONS

| Abbreviation | Expansion |
|---|---|
| A | Amoxicillin |
| *A* | *Aeromonas* |
| AP | Ampicillin |
| API 20E | Analytical Profile Index 20E biochemical identification technique |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| C | Chloramphenicol |
| CIP | Ciprofloxacin |
| CCL1 | Contaminant Candidate List 1 |
| CCL2 | Contaminant Candidate List 2 |
| CFU | Colony forming unit |
| CLSI | Clinical and Laboratory Standards Institute |
| DNA | Deoxyribonucleic acid |
| $dH_2O$ | Distilled water |
| E | Erythromycin |
| E value | Expect value |
| *E. coli* | *Escherichia coli* |
| EPA | Environmental Protection Agency |
| EPS | Extracellular Polymeric substances |
| HG | Hybridisation Group |
| ID | Identities |
| K | Kanamycin |
| KF | Cephalothin |
| L | Litre |
| MF | Membrane Filtration |
| Ml | Millilitre |
| NCCLS | National Committee for Clinical Laboratory Standard |
| OT | Oxytetracycline |
| PCR | Polymerase chain reaction |
| rDNA | Ribosomal deoxyribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| S | Streptomycin |
| spp. | Species |



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| | |
|---|---|
| SA | South Africa |
| subs | Subspecies |
| TM | Trimethoprim |
| TNTC | Too numerous to count |
| US | United States |
| USEPA | United states Environmental protection agency |
| WHO | World Health Organisation |
| WISA | Water Institute of Southern Africa |
| WRC | Water Research Commission |
| µm | Micro molar |
| - | Negative sign |
| + | Positive sign |
| °C | Degrees Celsius |
| > | Greater than |
| % | Percentage |
| α | Alpha |
| β | Beta |



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# ABSTRACT

**Aim of the study**: The aim of this study was to determine the prevalence and antibiotic resistance patterns of *Aeromonas* species from drinking water collected from rural households' drinking water containers in rural areas of Vhembe district, South Africa.

**Methods**: 100 drinking water and biofilm samples were collected from households' storage drinking water storage containers in Ngudza and Dzingahe rural villages in Vhembe district and analysed for the presence of *Aeromonas* spp. Membrane filtration technique was used for the isolation of *Aeromonas* species. API 20E biochemical tests were used to identify isolates to *Aeromonas* species level based on their biochemical characteristics and 16S rRNA gene sequencing method was used to confirm the identities of *Aeromonas* based on their genotypic content. Haemolysin production on blood agar was performed to determine haemolytic activity of the isolates. The virulence genes targeted to determine potential pathogenic ability of the isolates include *asa1*, *cylA, gelE and hyl*. The antibiotic resistance patterns of the isolates were determined using the Kirby Bauer disk diffusion method.

**Results**: *Aeromonas* prevalent in accordance to API 20E biochemical identification system was shown in 73 (44%) of the drinking water isolates and 45 (54%) of biofilm isolates. 15% of the drinking water and 23% biofilm *Aeromonas* prevalent was confirmed by 16S rRNA sequencing molecular identification method. β haemolytic activity was displayed in 57% of all isolates (of which 40% were biofilm and 17% were drinking water). α haemolytic activity was displayed in 43% (of which 20% were biofilm and 23% were drinking water) isolates. None of the isolates displayed gamma haemolytic activity in all confirmed isolates. Gelatinase gene (*gelE*) was detected in 67% out of which 20% was of drinking water and 47% of biofilm isolates. Aggregation substance (*asa1*) was observed in 50% out of which 17% was from drinking water and 33% biofilm from isolates. Cytolysin (*cylA*) and hyaluronidase (*hyl*) genes were not detected in both drinking water and biofilm isolates. Ampicillin, Amoxicillin and Erythromycin were highly resistant to all tested isolates. The most dominant antibiotic phenotype in this study was AP-A-C-E-KF-OT.

**Conclusion**: The presence of *Aeromonas spp.* and virulence factors from households' drinking water signifies a health risk to the community. High level of antibiotic resistance and even multidrug resistance patterns observed in this study to antibiotics commonly used for treatment of bacterial infections lessen the choice of antibiotics used for treatment of associated infections and thus increases public health risk.

**Keywords:** *Aeromonas* spp., drinking water, biofilms, and antibiotic resistance patterns.



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# Chapter 1
# GENERAL INTRODUCTION

## 1.1. BACKGROUND

*Aeromonas* species have been recognized as the etiologic agent of a variety of infectious complications in both healthy and immunocompromised individuals (Janda and Abbott, 2010). Members of the genus *Aeromonas* are generally noted by *Aeromonas hydrophila*, one of the human disease causative agents which is included in the *first and second contaminant candidate list (CCL1 and CCL2) by WHO* and the *US Environmental Protection Agency of potential waterborne pathogens* (Igbinosa et al., 2012; Minea et al., 2012).

Currently, *Aeromonas* species are considered as emerging waterborne and foodborne pathogens due to a vast number of published papers and reviews on *Aeromonas* as a human pathogen in recent years (Von Graevenitz and Mensch, 1968; Igbinosa et al., 2012). *Aeromonas* species are mainly associated with gastroenteritis, wound infection, and septicaemia in humans. Infections usually follow as a result of ingestion of contaminated water or food. It could also be as a result of trauma or contact with an open skin cut or injury in aquatic environment (Igbinosa et al., 2012).

*Aeromonas* infections in humans are principally associated with three frequently observed mesophilic species from both clinical and environmental samples. These species are *A. hydrophila*, *A. caviae*, and *A. veronii* subsp. *sobria* (Igbinosa et al., 2012). Increased antibiotic resistance patterns has been observed from environmental *Aeromonas* isolates (Piotrowska and Popowska, 2014). This is regarded as a threat to public health (Desai and Desai, 2014).

Species of the genus *Aeromonas* are ubiquitously found in aquatic environments. They have been isolated from various water sources such as rivers, lakes, ponds, seawater, groundwater, wastewater, sewage and also from municipal treated drinking water (USEPA, 2006). Recent studies have indicated that *Aeromonas* species isolated from untreated water sources can produce a wide range of virulence determinants that are linked with human infection. Their presence in drinking water is of concern as it poses a potential health risk (Mahdi et al., 2008). This is an emerging health problem, particularly for immunocompromised individuals as infections to these individuals can results in severe manifestations (Parker and Shaw, 2011;



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Tomas, 2012). Drinking water is essential to sustain a healthy life, and every effort should be made to supply safe drinking water to consumers. The World Health Organization recommended that water meant for drinking and household purposes should be free from any pathogenic organism that might pose a health risk to consumers up to the point of delivery (WHO, 2008). However, drinking water is currently thought to be one of the reservoirs of *Aeromonas* species, since the genus *Aeromonas* occur naturally in almost all types of fresh water (WHO, 2011). As a result, the wide distribution nature of *Aeromonas* spp. in aquatic environments increases their transmission opportunities to humans through contaminated water or direct human contact (Janda and Abbott, 2010; Odeyemi and Ahmad, 2013).

In many rural areas of South Africa, potable water for domestic use is supplied to communities at communal taps (Nala et al., 2003). However, such communal taps are located some distance from the home, and thus require collection and transport from the source and subsequent storage of water within the household storage containers (Wright et al., 2004). Therefore water for domestic use, including drinking water is likely stored in bulk storage containers. These are normally 20-25 litre narrow mouth plastic storage containers with screw caps (Nieuwoudt and Matthews, 2005). Due to availability insecurity rural communities are forced to store water for domestic use in the home for a period of 24 hours or longer before use (Trevett et al., 2004).

Previous studies have shown that potable water supplied from the public standpipe becomes microbiologically contaminated during handling, poor storage conditions, and unhygienic practices at home (Gundry et al., 2004; Nieuwoudt and Matthews, 2005). There are concerns regarding in-house microbial contamination due to lack of proper cleaning and storage practices of drinking water storage containers in rural areas. As a result, microbial contamination renders the drinking water stored in household's containers unsafe for human consumption (Potgieter, 2007).

Hygienic condition of the water storage containers is believed to be the major factor leading to the deterioration of stored water (Maraj et al., 2006). However, formation of biofilm within these storage containers is also believed to be another factor that lead to the deterioration of stored drinking water (Jagals et al., 2003). Literature indicated that microorganisms attach on the surfaces of such containers during storage and develop into biofilms even though there is a very low concentration of organic carbon in water. Such attached bacteria can detach from the surface walls leading to continuous contamination of the water phase during the filling and empyting of the container (Momba and Kaleni, 2002). A large variety of different opportunistic and pathogenic heterotrophic bacteria such *Aeromonas* spp., *Legionella* spp., *Pseudomonas*



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spp., *Mycobacteria* spp., *Campylobacter* spp., *Klebsiella* spp., *Helicobacter pylori*, *Salmonella* and *E. coli* have been isolated from biofilms in drinking water (Momba and Kaleni, 2002; September et al., 2007).

Water supply through improved piped systems in some rural areas faces major problems in terms of operation and maintenance (Momba et al., 2005). Thus many villages that are, during census periods, counted as served with potable drinking water, no longer have functional water supplies (Momba et al., 2005). As a result, the majority of communities in such rural areas are forced to obtain water for drinking and food preparation from untreated water sources which include rivers, streams, lakes, ponds, shallow wells, and springs (Ghenghesh et al., 2008; Molelekwa et al., 2014). Consumption of such water from untreated sources present a health risk, as it might expose the consumers to potential pathogenic bacteria including species of *Aeromonas*.

In the past few years, resistance to the most commonly used antibiotics for treating *Aeromonas* infections has been observed in several countries (Janda and Abbott, 2010: Saavendra, 2012). Currently, resistance to commonly used antibiotics is considered as another characteristic of *Aeromonas* species (Janda and Abbott, 2010; Figueira et al., 2011) and this signifies difficulties in the treatment of *Aeromonas* infections. Furthermore, *Aeromonas* detected from drinking water in a study by Figueira et al, (2011) in Northern Portugal were confirmed as relevant agents for spreading antimicrobial resistance in the aquatic environment. For this reason, it has been concluded that *Aeromonas* can serve as a vehicle for resistance transfer in an aquatic environment to water consumers (Figueira et al., 2011).

## 1.2. STUDY RATIONALE

*Aeromonas* species are presently considered as important human pathogens which possess a variety of emergent virulence factors that are associated with both gastrointestinal and extra-intestinal illnesses in humans (Kivanc et al., 2011; Robertson et al., 2014). Although the majority of studies in the past few years have been focused on clinical sources, findings from recent studies, confirmed the role of water as a vehicle of *Aeromonas* diseases (Khajanchi et al., 2010; Correia, 2013). It has been indicated that environmental *Aeromonas* isolates are resistant to new antibiotics which are commonly used in medicine and this can lead to a potential risk to public health (Saavendra, 2012). *Aeromonas* spp. are potential waterborne pathogens and it is therefore important to consider their regulation in drinking water (Correia,



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2013; Pandove et al., 2013). *Aeromonas* species have been observed in chlorinated drinking water as a result of regrowth and multiplication in water distribution systems (Trower et al, 2000; Igbinosa et al., 2012). This is due to the fact that several studies have indicated the ability of *Aeromonas* spp. to survive and to form biofilms in drinking water distribution systems where they may become resistant to chlorination or common disinfectants (Igbinosa et al., 2012; Desai and Desai, 2014). Therefore the presence of these species in potable water increases resistance to chlorine and this in turn increases health risk to humans (Subashkumar et al., 2006).

A large number of *Aeromonas* spp. was detected from a variety of biofilms within drinking water distribution systems in South Africa (September et al., 2007). Biofilm in water distribution system is defined as a complex mixture of bacterial species, organic and inorganic material accumulated amongst a microbial produced organic polymer matrix attached to the inner surface of the distribution system (USEPA, 2002). Biofilms generally grow in association to all surfaces in contact with non-sterile water such as in drinking water processing, storage and distribution systems (Flemming, 2002; Flemming, 2011). The data from a study carried out in rural communities of South Africa revealed that microorganisms are able to attach on the inner surface wall of drinking water storage containers (Momba and Mngumevu, 2000).

The occurrence of biofilms in drinking water is seen as a microbial reservoir for further contamination and multiplication of opportunistic bacterial pathogens such as *Aeromonas* and *Pseudomonas* in drinking water (Szewzyk et al, 2000; Wingender and Flemming, 2011). Biofilms in drinking water systems and within storage deteriorate the microbiological water quality and thus threatens health risk to the water consumers (LeChevallier and Au, 2004). Literature also indicates that biofilms are one of the factors that promote biofilm-associated bacteria to become resistant to antibiotics. Resistance of biofilm producing bacteria to antibiotics has been suggested to be the most significant source of non-effective therapy of biofilm associated bacterial infections (Ciofu and Tolker-Nielsen, 2011).

A number of virulence factors such as aerolysin, haemolysin, protease, lipase, gelatinase and DNase and other virulence extracellular products produced by *Aeromonas* spp. play an important role in the development of disease in humans (Igbinosa et al., 2012). As a result *Aeromonas* have been the topic of recent renewed interest, principally because of increased awareness about their role in human illnesses (Trower et al, 2000). The ubiquitous nature of *Aeromonas* in aquatic environments indicates that their interactions with humans are continual



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and unavoidable enabling their opportunistic pathogenicity, which results in human infections (Parker and Shaw, 2011). Therefore it is of public health significance to identify potentially pathogenic *Aeromonas* spp. from drinking water and their antibiotic resistance patterns to ensure prevention of human illness and proper treatment to associated infections (Trower et al., 2000).

It has been observed from previous studies that the majority of *Aeromonas* strains isolated from aquatic environments showed continuous and increased drug resistance and multidrug-resistance profiles (Huddleston et al., 2006). Increased drug resistance presents a significant threat to the management of most commonly *Aeromonas* mediated diarrhoeal illnesses (Mahdi et al., 2008). Antibiotic resistance patterns of both clinical and environmental isolates are needed for implementation of the most effective antibiotics for the treatment of *Aeromonas* associated infections in humans. However, little data are available on antibiotic resistance patterns of bacterial isolates from drinking water used for domestic purposes, especially for drinking in rural communities of Vhembe district. Therefore there is a need to uncover resistance patterns of potential *Aeromonas* human pathogens from drinking water isolates in rural household storage water containers.

Several pathogenic *Aeromonas* spp. are known to cause human infections through either ingestion of water or food. However, with insufficient information about the prevalence of *Aeromonas* spp. in drinking water storage containers and their antibiotic resistance, little can be done to control their associated infections. There is a need to regularly monitor water sources for prevalence of potential pathogenic *Aeromonas* species threatening water safety. Therefore this study has focused on determining the prevalence and antibiotic resistance patterns of *Aeromonas* spp. at the point of use from drinking water storage containers in Vhembe district under Limpopo province of South Africa.

## 1.3. SIGNIFICANCE OF THE STUDY

People need drinking water daily for proper health and wellness. Most people in rural areas collect water from public standpipes and also from natural water sources such as wells, springs and rivers and then store it in specific containers in their households for their daily water needs. Assessment of prevalence of *Aeromonas* species from drinking water in household storage containers is important to estimate the amount of potential pathogenic *Aeromonas* spp. being consumed at the point of use. Sufficient data on prevalence of



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*Aeromonas* species in water used for domestic purpose can be used or analysed microbiologically to draw helpful measures required to prevent or reduce the health risk associated with potential pathogenic *Aeromonas* species.

The knowledge of antibiotic resistant patterns to most commonly used antibiotics for treatment of infections associated with drinking water *Aeromonas* isolates is of human health importance. Given the fact that *Aeromonas* species are resistant to low chlorine levels, especially in water stored for a long period in storage water containers where chlorine levels decline over time and allow these bacteria to grow and multiply. Therefore data from the present study will be of help in rural areas where most people experience a challenge of access to safe drinking water and are forced to use water in storage containers, regardless of long storage periods. Furthermore, the updated data on antibiotic resistance patterns of *Aeromonas* isolates from drinking water could be used to verify the most effective antibiotics for treatment of *Aeromonas* associated infections.

## 1.4. OBJECTIVES OF THE STUDY

Primary Objective:

To determine the prevalence and antibiotic resistance patterns of *Aeromonas* species from drinking water collected from rural households' drinking water containers in rural areas of Vhembe district, South Africa.

Secondary Objectives:

- To isolate and purify presumptive positive *Aeromonas* spp. from drinking water and biofilms samples inside household water storage containers.
- To identify *Aeromonas* spp. using biochemical identification tests and 16S rRNA gene sequencing.
- To assess the virulence capabilities of *Aeromonas* isolates using blood agar and PCR for detection of selected genes.

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- To uncover the antibiotic resistance patterns of *Aeromonas* positive isolates using Kirby-Bauer disk diffusion method.

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# Chapter 2
# LITERATURE REVIEW

## 2.1. Introduction

Initially, *Aeromonas* species were recognized only as disease-causing bacterial pathogen in fish and other coldblooded species (Janda and Abbott, 2010). However in 1968, von Graevenitz and Mensch reviewed 30 cases of *Aeromonas* human infections which has established *Aeromonas* spp. as significant human pathogens (von Graevenitz and Mensch, 1968; Igbinosa et al., 2012). To date, *Aeromonas* infections in humans are universally caused by the following species; *A. hydrophila*, *A. caviae*, *A. veronii biovar sobria*, *A. jandei*, *A. veronii biovar veronii*, *A. schuvertii* and *A. trotta*. However, only three mesophilic species are principally associated with 85% of human infections and such species include *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas veronii* subsp. *sobria* (Igbinosa et al., 2012).

*Aeromonas* are currently considered as emerging pathogens and have been implicated in clinical cases of diarrhoea, due to their recognized virulence ability to cause diseases in humans. Their wide distribution in aquatic environment supports the fact that infections are mainly acquired through ingestion of contaminated water or food, although there is no major outbreak that has been reported (Sen and Rodgers, 2004). The wide distribution of *Aeromonas* species in aquatic environment has been recognized as a potential health risk, and some countries have adopted *Aeromonas* as an additional indicator of water quality (Senderovich et al., 2008). *Aeromonas* is currently included by the World Health Organization and US Environmental Protection Agency in the Contaminant Candidate List of emerging pathogens in drinking water (Minea et al., 2012).

*Aeromonas* strains isolated from water have been indicated to possess virulence factors, such as adhesins, haemolysins and cytotonic enterotoxins, that are involved in *Aeromonas* associated human diseases. Pathogenic *Aeromonas* spp. are responsible for gastroenteritis, septicaemia, and wound infections in humans (Senderovich et al., 2008). It has been recognized that *Aeromonas* can lead to infection in both immune-competent and immunocompromised individuals (Parker and Shaw, 2011). Nearly all the studies in developing countries on the prevalence of *Aeromonas* spp. in cases of diarrhoea have been reported from children, immunocompromised individuals and very few from mixed population (Ghenghesh et al., 2008).



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Although gastrointestinal infections are usually self-limiting with individuals recovering without treatment, antibiotic therapy is required for infants or immunocompromised patients with prolonged infection (USEPA, 2006), as well as severe and unresponsive cases of *Aeromonas* gastroenteritis and also extra intestinal infections (Igbinosa et al., 2012). However, currently increase in antibiotic resistances among *Aeromonas* species have been reported in several developing countries (Saavendra, 2012).

## 2.2. The biology and taxonomy of the genus *Aeromonas*

Members of the genus *Aeromonas* belong to the family Aeromonadaceae within the class of Gamma-proteobacteria (Abbott et al., 2003; Ghenghesh et al., 2008; Janda and Abbott, 2010). The first classification of the genus *Aeromonas* was based upon phenotypic features and revealed only 4 species namely, *A. hydrophila*, *A. caviae*, *A. sobria* presently named as *A. veronii* biovar sobria and A. *salmonicida* (Ghenghesh et al., 2008). Later on, the new genotypic methods such as DNA hybridisation groups (HG) (Ghenghesh et al., 2008), the ribosomal gene 16S (small subunit) molecular technique (Martinez-Murcia et al., 1992) and Restriction Fragment Length Polymorphisms (RFLP) of the 16S rDNA amplified by polymerase chain reaction (Figueras et al., 2000) were introduced and established for the successful classification of the species within the genus *Aeromonas*.

The establishment of DNA-DNA hybridization molecular technique has allowed identification of 17 hybridization group or genospecies and 14 phenospecies within the genus *Aeromonas* (Ghenghesh et al., 2008). The species were identified as *A. hydrophila* (HG1), *A. bestiarum* (HG2), *A. salmonicida* (HG3), *A. caviae* (HG4), *A. media* (HG5), *A. eucrenophila* (HG6), *A. sobria* (HG7), *A.* veronii biotype sobria (HG8), *A. jandaei* (HG9), *A.* veronii biotype veronii (HG10), *A. schubertii* (HG12), *A. trota* (HG14), *A. allosaccharophila* (HG15) , *A. encheleia* (HG16) , and *A. popoffii* (HG17) ; and two unnamed DNA-hybridisation groups, *Aeromonas spp.* (HG11) and *Aeromonas* Group 501 (HG13), without association with an actual species. In addition, three species without the hybridisation group namely, *A. culicicola*, *A. simiae*, and *A. molluscorum* have been described (Ghenghesh et al., 2008; Igbinosa et al., 2012).

The genus *Aeromonas* is divided into two principal groups based on their growth and biochemical characteristics namely, mesophilic and psychrophilic group (Ghenghesh et al., 2008; Janda and Abbott, 2010). The first mesophilic group consists of motile species that grow well at an optimum temperature range of 35-37°C. Most strains within mesophilic group



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possess mainly a single polar flagella for motility, however, some species produce lateral and peritrichous flagella (Janda and Abbott, 2010; Igbinosa et al., 2012). The mesophilic group is much larger; designated *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* species which are responsible for a range of human infections and thus this group is of human health significance (Percival et al., 2004; Janda and Abbott, 2010). In most cases, species associated with human infections were recognized to be *A. hydrophila* which also explain the reason the mesophilic group is typified by the species *A. hydrophila*. The second psychrophilic group consists of non-motile species, which grow well at an optimum temperature range of 22-28°C. The psychrophilic group is a small group; designated only *Aeromonas salmonicida* species that infects reptiles and fish. However, some species within the genus *Aeromonas* can also grow at a temperature that range from 0-45°C. This feature enables them to survive in a wide distribution of environment which in turn increases the risk of human exposure (Ghenghesh et al., 2008; Igbinosa et al., 2012).

Members of the genus *Aeromonas* are rod-shaped with rounded ends which measure 0.3-1.0µm by 1.0-3.5µm, facultatively anaerobic, and non-spore forming Gram-negative bacteria. Species of *Aeromonas* are oxidase positive, catalase positive, indole positive, and chemoorganotrophic bacterial strains that have the ability to utilize a variety of different sugars and carbon as a source of energy (Percival et al., 2004; Ghenghesh et al., 2008; Igbinosa et al., 2012). *Aeromonas* species are able to catabolize glucose while producing acids as well as gases and ferment maltose, D-galactose, and trehalose, however they are unable to ferment xylose, sorbose, erythritol, adonitol, dulcitol (Parker and Shaw, 2011; Tomas, 2012). In addition, *Aeromonas* species are able to reduce nitrates to nitrites (Tomas, 2012). All species within the genus *Aeromonas* are able to tolerate the pH that range from 5-10 (Percival et al., 2004) and their optimum sodium chloride range from 0-4% (Igbinosa et al., 2012).

## 2.3. Poor access to potable water in rural areas of South Africa

In many rural communities of South Africa, drinking water is usually supplied through public standpipes systems. Even so, due to the long distance from the communities' households to the drinking water sources, inhabitants require household containers to be used for collection and storage of drinking water in the home. However, it is well known that microorganisms can attach on the surface walls of such containers during storage where they can lead to deterioration of stored water quality. It has been indicated that re-growth of *Aeromonas* in biofilm in containers, can results in an increase in the number of opportunistic bacteria in stored drinking water (Momba and Kaleni, 2002; Levy et al., 2008).



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Presently, it is noted that majority of people in rural communities under Vhembe district of South Africa lack well developed water infrastructures. Most of the infrastructure that were planted by the government are no longer functioning properly and in most cases it takes a long period for the government to fix the water infrastructure back to its normal functioning. As a result, water supply through improved piped systems in many rural villages is said to face major problem in terms of operation and maintenance and majority of people counted as served, no longer have functional water supply. During the period of improper functioning of the water infrastructure people in these communities rely on untreated raw water sources such as rivers, streams, lakes, ponds, springs, well water and also rely on rain water (Mololekwa et al., 2014). Water from such sources are not safe for human consumption as they are usually faecal contaminated and also because they were reported to be of poor microbiological quality by previous studies done in South Africa (Obi et al., 2007; Ghenghesh et al., 2008).

Furthermore, previous studies have reported that the drinking of untreated water is the most likely manner of getting *Aeromonas* infections (Ghenghesh et al., 2001; Massa et al., 2001; Di Bari et al., 2007). Enteropathogenic *Aeromonas* have been isolated from untreated water sources including those used for drinking in developing countries (Ghenghesh et al., 2008). According to literature *Aeromonas* can produce a wide range of virulence determinants that are linked with human infection, and therefore their presence in drinking water is of potential health risk (USEPA, 2006; Mahdi et al., 2008).

## 2.4. Transmission of *Aeromonas* spp. through drinking water

*Aeromonas* species are known to occur naturally in aquatic environments and were frequently isolated from drinking water in previous studies. They are found worldwide in fresh water, surface water, ground water, and wastewater, and have been isolated in chlorinated drinking water, non-chlorinated drinking water, and bottled mineral water. They are also found in soil and faeces of aquatic and terrestrial animals, including humans. *Aeromonas* were also isolated in a variety of foods such as fish and other sea foods, vegetables, raw milk, and processed food products (USEPA, 2006). These organisms do not occur in water with a very high salinity, geothermal springs or extremely polluted rivers (Ghenghesh et al., 2008).

Ingestion of untreated water and raw foods remain the primary route of transmission for *Aeromonas* causing waterborne and food-borne illness. Most available data suggest that the majority of *Aeromonas* responsible for human infections are acquired through contact with



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contaminated drinking water or through the ingestion of foods exposed to *Aeromonas* bacterial species. In addition to these major transmission routes, *Aeromonas* can also be acquired through recreational activities such as boating, fishing, and swimming that can lead to infection through open skin or wound contact (USEPA, 2006). Exposure to water contaminated with *Aeromonas* spp. through either ingestion or skin contact can results in skin inflammation and gastroenteritis, mainly at increased immunocompromised individuals (Minea et al., 2012).

According to previous studies, a causal relationship exists between the strains of *Aeromonas* found in drinking water and those isolated from cases of human diarrhoea (Khajanchi et al., (2010; Parker and Shaw, 2011). Data obtained from a study by Khajanchi et al., (2010) indicated similar virulence characteristics from water and clinical isolates, suggesting possible transmission of *Aeromonas* species from water to humans. Findings from a study done by Parker and Shaw, (2011) also demonstrate evidence of successful colonization and subsequent infection of a number of *Aeromonas* species after transmission from water to humans (Parker and Shaw, 2011).

Seasonality seems also to play a role in *Aeromonas* associated infections. *Aeromonas* associated gastroenteritis cases were found to be higher in number during the summer months in correlation with increased numbers of *Aeromonas* in the water systems (Parker and Shaw, 2011). An increase in number of *Aeromonas* during summer increases opportunities for exposure to these bacteria during warmer months and thus increases the chance of transmission from water to humans (Janda and Abbott, 2010).

## 2.5. Biofilms and *Aeromonas* regrowth in treated drinking water

Biofilms can be defined as group of microorganisms that attach and grow on any solid surfaces in contact with non-sterile water enclosed in a matrix of extracellular polymeric substances (EPS) (Donlan, 2002; Flemming, 2011). Most enterobacteria including *Aeromonas* species possess the ability to form biofilms on many surfaces in contact with water (Kostakioti et al., 2013). The process of biofilm formation basically involves five stage processes which includes, initial attachment of bacterial cells to wet surface. This is followed by the production of extracellular polysaccharides where in attached cells begin to reproduce and form microcolonies on the surface and proceed to produce a polymer matrix around the microcolonies. Successful attachments lead to the development of the mature biofilm and finally dispersion of bacterial cells from the biofilm to colonize other surfaces within the same aquatic environment (Stoodley et al. 2002; Lasa, 2006).



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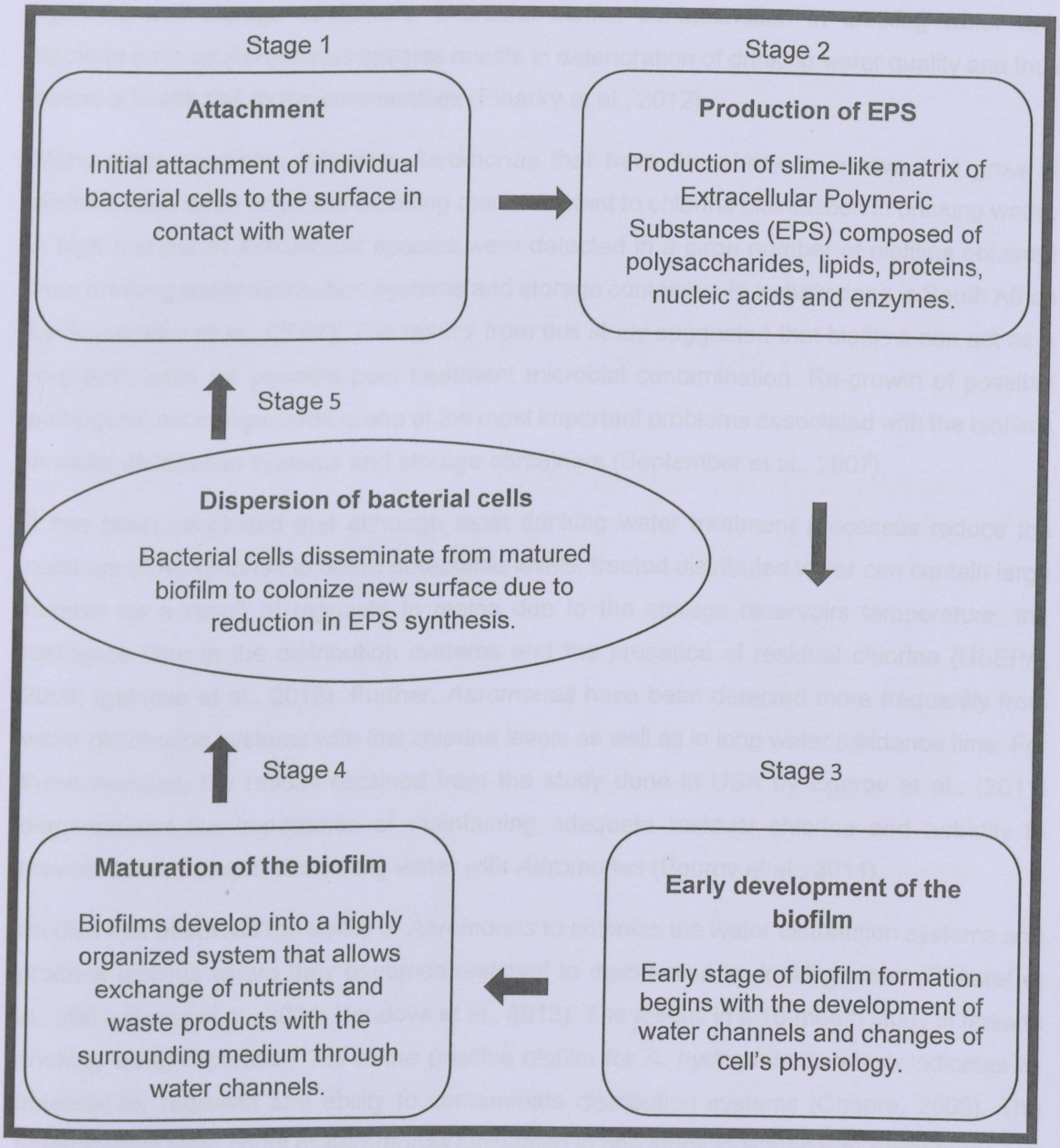



**Figure 2.1:** Flow diagram indicating stages of biofilm development.

*Aeromonas* has demonstrated the ability to grow and survive in biofilms within the treated drinking water distribution systems. Biofilms are known to protect several microorganisms including *Aeromonas* from environmental stress conditions and water disinfectant such as chlorine. As a result, the presence of biofilms in drinking water distribution systems and storage containers support the regrowth of *Aeromonas* and other enterobacteriaceae bacterial species in drinking water (Elhariry et al., 2012; Igbinosa et al., 2012). Biofilm is considered as one of the most important sources of microbial contamination in drinking water distribution



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systems and storage containers. Microbial biofilm contamination in drinking water with bacteria such as *Aeromonas* species results in deterioration of drinking water quality and thus poses a health risk to the communities (Elhariry et al., 2012).

Many microorganisms including *Aeromonas* that have the ability to survive and grow in biofilms have been observed as being more resistant to chlorine disinfection in drinking water. A high number of *Aeromonas* species were detected in a large number of biofilms obtained from drinking water distribution systems and storage containers in a study done in South Africa by September et al., (2007). The results from this study suggested that biofilms can act as a re-growth zone for possible post treatment microbial contamination. Re-growth of possible pathogenic microorganisms is one of the most important problems associated with the biofilms in water distribution systems and storage containers (September et al., 2007).

It has been concluded that although most drinking water treatment processes reduce the numbers of *Aeromonas* to below detectable levels, treated distributed water can contain large number as a result of regrowth in mains due to the storage reservoirs temperature, the residence time in the distribution systems and the presence of residual chlorine (USEPA, 2006; Igbinosa et al., 2012). Further, *Aeromonas* have been detected more frequently from water distribution systems with low chlorine levels as well as in long water residence time. For these reasons, the results obtained from the study done in USA by Egorov et al., (2011) demonstrated the importance of maintaining adequate residual chlorine and turbidity to prevent contamination of drinking water with *Aeromonas* (Egorov et al., 2011).

Studies has observed the ability of *Aeromonas* to colonize the water distribution systems and produce biofilms where they becomes resistant to disinfection in drinking water (Chauret et al., 2001; Bomo et al., 2004; Pandove et al., 2013). The results in a 16-month study in Indiana drinking water reported 7.7% of the positive biofilm for *A. hydrophila* in, which indicates its potential for regrowth and ability to contaminate distribution systems (Chopra, 2008). The heterotrophic plate count of *Aeromonas* calculated in one study indicated that *Aeromonas* can make up a total of 1-27% in samples of finished drinking water, implicating drinking water as a possible source of infection (Parker and Shaw, 2011). In addition, evidence from surveys in the Netherlands also indicates that re-growth of *Aeromonas* can occur in drinking-waters derived from anaerobic groundwater sources (USEPA, 2006).

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## 2.6. Pathogenicity of *Aeromonas* species

*Aeromonas* species possess a number of virulence factors which generally comprise of cell-associated features and extracellular factors that plays important roles involved in pathogenesis (USEPA, 2006). Amongst the virulence factors involved in pathogenic potential of *Aeromonas* spp. include those that promote attachment, allow colonization, immune evasion and proliferation in the host cells to initiate infections (Igbinosa et al., 2012). Although it is not completely understood how these virulence factors cause infections to humans, it is noted that they works together multi-factorially and multi-functionally to cause infections in humans (Pandove at al., 2013).

Cell-associated factors or structural components include flagella, pili (fimbriae), adhesins outer membrane proteins, capsule, S-layers, lipopolysaccharide, and surface α-Glucan (USEPA, 2006; Igbinosa et al., 2012 and Tomas, 2012). Flagella, pili and adhesins contribute to *Aeromonas* pathogenesis through attachment and entry into the host cells. The outer membrane proteins produced by *Aeromonas* species mediate bacterial adherence into the host cells and also helps in multiplication of pathogenic bacteria inside the host cell (USEPA, 2006; Tomas 2012). The production of capsules and S-layers by *Aeromonas spp.* contributes in their resistance to host defences mechanisms through their ability to prevent phagocytosis, protection against complement and instead favour interactions to other bacteria and host tissues. The presence of lipopolysaccharide has been described to facilitate adherence to the epithelial cells and thus protect host cells from immune response. In addition to *Aeromonas* pathogenicity, the surface α-glucan produced by mesophilic *Aeromonas* strains plays an important role in biofilm formation (Tomas, 2012).

Extracellular factors involve a large number of extracellular enzymes such as proteases, lipases, amylases, chitinases, nucleases, and gelatinases as well as enterotoxins, haemolysins, and sideophores. Generally, some of these extracellular enzymes contribute to pathogenicity by promoting invasion into the host cell which is one of the significant steps for initiation of infection in host cell. However, most of the extracellular enzymes have been described to actively degrade a variety of complex protein, polysaccharide, mucopolysaccharide, and lipid-containing molecules and thus contribute to the establishment of infection by overcoming the initial host defense (USEPA, 2006; Tomas, 2012; Desai and Desai, 2014). Furthermore, a study done by Correia in (2013) discovered the ability of *Aeromonas* strains to express a variety of extracellular virulence factors involved in the



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infection process similar to other enteropathogen, thus showing their role to damage host tissues as well as to evade the host defense system (Correia, 2013).

In addition to extracellular factors, *Aeromonas* species secrete two main types of enterotoxins referred to as cytotoxic (or cytolytic) and cytotonics. Cytotoxic enterotoxins have been described to provoke degeneration of crypts and villi of the small intestine and these usually result in diarrheal infections in humans. Enterotoxins such as the well-known virulence gene Act also known as an aerolysin-related pore-forming toxin is responsible for the hemolytic, cytotoxic, and enterotoxic activities of pathogenic *Aeromonas* species (Tomas, 2012). Furthermore, some investigators observed that *Aeromonas* induced gastroenteritis is due to an enterotoxin which is cytotoxic in nature but Stelma et al., (1986) reported aerolysin to be the main virulence factor involved in intestinal disorders (Pandove et al., 2013). The haemolysin factor also known as aerolysin produced by some *Aeromonas* spp. has been shown to have both hemolytic and enterotoxic activity which are responsible for major human infections (Ghenghesh et al., 2008).

The production of haemolytic toxin which is one of the cytotoxic enterotoxins is considered to be evidence of the pathogenic potential of *Aeromonas* species. Haemolytic activity involves pore formation in the membrane of the target cell which leads to the degradation of the host red blood cells (Tomas, 2012; Yadav et al., 2014). *Aeromonas* spp. produce two classes of hemolysins without enterotoxic properties: referred to as α-hemolysins and β- hemolysins. The α-hemolysins are synthesized in the stationary bacterial growth phase and lead to reversible cytotoxic effects and incomplete destruction of red blood cells. On the other hand, the β-hemolysins are pore forming toxins that are usually synthesized in the exponential bacterial growth phase which lead to osmotic lysis and complete destruction of red blood cells (Tomas, 2012).

The production of a wide array of virulence factors by *Aeromonas* species is indicative of their potential to cause severe diseases in humans. It has been suggested that the presence of any virulence gene in strains of *Aeromonas* isolated from water should be carefully examined; as such genes could be expressed better in a human host, which could lead to devastating outcomes (Khajanchi et al., 2010). These virulence factors are useful in distinguishing between potentially pathogenic and non-pathogenic strains. However, Correia (2013) has shown that infections by potentially pathogenic *Aeromonas* spp. may not always cause



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disease to healthy individuals and also due to low infectious dose of the microorganism (Correia, 2013).

## 2.7. Incidence and clinical manifestations associated with pathogenic *Aeromonas* species

*Aeromonas* species have been reported as causative agents of a wide range of diseases in humans (Ghenghesh et al., 2008). *Aeromonas* are responsible for gastrointestinal and extra-intestinal diseases, ranging from mild illnesses to life-threatening conditions. Major human *Aeromonas* infections include gastroenteritis, wound infections, muscle infections, septicaemia or bacteraemia, and skin diseases. Both *Aeromonas* gastrointestinal and extra-intestinal infections are now known to occur in both healthy and immunocompromised individuals (USEPA, 2006). It is however noted that *Aeromonas* infections in the immunocompromised are usually more severe than in the immune-competent individuals (Ghenghesh et al., 2008).

Gastroenteritis is the most common human illness associated with *Aeromonas* spp. *Aeromonas* associated gastroenteritis usually occur in the form of self-limiting liquid diarrhoea to a more severe and invasive diarrhoea, which is a problem particularly for young children, the elderly and immunocompromised patients. However, illness may also occur in healthy individuals of any age (Trower et al, 2000; Tomas, 2012). *Aeromonas* gastroenteritis usually manifests itself in three ways which include an acute self-limiting, watery diarrhoea, dysentery-like mucus and bloody diarrhoea, and chronic diarrhoea (Parker and Shaw, 2011).

In addition to gastroenteritis, dehydration may follow the associated symptoms in severe cases (Ghenghesh et al., 2008). Aerolysin gene in *Aeromonas* strains has been reported as the main cause of gastrointestinal infections in humans, although other researchers have discovered that enterotoxins which are cytotoxic in nature are responsible gastrointestinal infections in humans. Diarrhoea is the main infections caused by these bacteria in both immunocompetent and immunocompromised individuals (Pandove at al., 2013).

Extra-intestinal infections of environmental origins arise directly from water or soil contact, or indirectly by ingestion and bacteria dissemination of *Aeromonas* from the gastrointestinal tract (Igbinosa et al., 2012). A variety of *Aeromonas* extra-intestinal infections include wound infections, septicaemia, respiratory tract infections and less frequently meningitis, peritonitis, eye infections and haemolytic uremic syndrome (Tomas, 2012). Wound infections are the



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second most common type of *Aeromonas* associated extra-intestinal infection in humans (Parker and Shaw, 2011).

Wound infections usually occur as a result of exposure to contaminated fresh water through swimming and boating accidents, and fishing-hook accidents (Huddleston et al., 2006). However, in healthy individuals wound infections usually occur through penetration to injury in water or soil, where *Aeromonas* species are present in high numbers (Parker and Shaw, 2011). In some cases where *Aeromonas* wound infection progress quickly and becomes systemic, infection can be fatal. In *Aeromonas spp.* conditions of wound infections include cellulitis, myonecrosis, and ecthyma gangrenosum and they need antibiotics for treatment (Huddleston et al., 2006; Parker and Shaw, 2011).

Septicaemia occurs as a result of *Aeromonas* dissemination from intestinal tract to systems of circulation (Tomas, 2012). *Aeromonas* septicaemia infection results in diarrhoea, fever, chills and abdominal pain as main symptoms. Septicaemia infection is generally observed in immunocompromised patients with predisposing medical conditions. Septicaemia has a mortality rate of over 90% in patients suffering from severe wound infection. *Aeromonas* species have also been linked to small number of respiratory infections case in both healthy and immunocompromised individuals (Parker and Shaw, 2011). Respiratory tract infections associated with *Aeromonas* include epiglottis and pneumonia (Tomas, 2012). Such respiratory infections usually occur as a result of near-drowning or inhalation of water whilst swimming (Parker and Shaw, 2011).

*Aeromonas* infection remains among those infectious diseases of potentially serious threat to public health (Igbinosa et al., 2012). However the exact incidence of *Aeromonas* infections on a global basis is unknown, since many cases go undetected and some are not reported (Janda and Abbott, 2010). Even though there have not been reported *Aeromonas* water-associated outbreaks in developing countries (Ghenghesh et al., 2008) there are evidence that suggest that routine endemic exposure to waterborne and food-borne outbreaks may occur more frequently than originally perceived due to the underestimation of the incidence of *Aeromonas* infections in developing countries (Igbinosa et al., 2012).

Some studies have shown that some motile *Aeromonas* species are becoming food and waterborne pathogens of increasing importance. They have been associated with several food-borne outbreaks and are increasingly being isolated from patients with traveler's diarrhoea (Igbinosa et al., 2012). The use of microbiologically unmonitored drinking water and



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poor hygienic living conditions were suggested to be responsible for the *Aeromonas*-associated acute diarrhoea outbreak that occurred in Sao Bento do Una, Pernambuco, Brazil reported by Hofer et al., (2006) (Ghenghesh et al., 2008). Diarrhoea is the most common manifestation associated with these outbreaks (Igbinosa et al, 2012).

Findings of *Aeromonas* infections incidence have been documented in several countries. In Limpopo province of South Africa, Obi and Bessong reported the isolation of *Aeromonas* spp. from 13.3% HIV patients with chronic diarrhoea in rural communities (Obi and Bessong, 2002). *A. hydrophila* was isolated as the sole enteropathogen from 80% diarrhoeal and 20% asymptomatic cases (Aslani and Alikhani, 2004). *A hydrophila* was found having the highest prevalent rate of 13.0% amongst other bacterial species identified from collected stool samples during the seasonal episodes of gastroenteritis in Nsukka, Nigeria (Nzeako et al., 2002). In Coimbatore of India, the presence of pathogenic *Aeromonas hydrophila* was reported in 9.7% of diarrheal stool samples tested of children less than 6 years (Subashkumar et al., 2006). In Chennai, India, Vila et al., (2003) reported 4.7% of *Aeromonas hydrophila* in stools samples analysed (Vila et al., 2003). Moreover, In India, Sinha et al., (2004) reported *Aeromonas* in 6.5% of all patients (Sinha et al., 2004). In Hong Kong, Chan et al., (2003) also documented 6.9% of adult patients with acute diarrhoea. The isolation rate of 64% *Aeromonas* isolates was reported among children with gastroenteritis in El-Minia governorate in Egypt (Mahdi et al., 2008). All these cases suggest or point out that *Aeromonas* spp. might have played an important role as causative agent of human infections.

## 2.8. Antibiotic resistance among *Aeromonas* species.

Although it has been indicated that antibiotics are not necessary to treat gastrointestinal *Aeromonas* infections in healthy individuals due to the fact that patients usually recover without treatment, antibiotics are considered for chronic gastrointestinal disease and extra-intestinal infection. Antibiotics are also required to treat severe and unresponsive cases of *Aeromonas* gastroenteritis especially in immunocompromised patients (USEPA, 2006). However, several developing countries reported a high rate of resistance to commonly used, cheap oral antibiotics among enteric pathogenic bacteria isolates from water environment in the last two decades (Ghenghesh et al., 2008).

Antibiotics are generally employed prophylactically and therapeutically to treat microbial diseases in humans and animals. However, in recent years the development of resistant as well as multidrug resistant pathogens has become a major health problem in terms of



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chemotherapy in many countries (Subashkumar et al., 2006; Orozova et al., 2008). According to the results obtained in a study done by Figueira et al, (2011) in Northern Portugal; *Aeromonas* detected in drinking water were confirmed as relevant agents for spreading antimicrobial resistance in the environment. It has also been concluded in this study that *Aeromonas* can serve as vehicle of resistance transfer in aquatic environment to the water consumers (Figueira et al., 2011).

Currently, resistance to commonly used antibiotics is considered as another characteristic of *Aeromonas* species (Janda and Abbott, 2010; Figueira et al., 2011). *Aeromonas* species have been shown to possess integrons, which are believed to be responsible for the resistance to commonly used antibiotics such as tetracyclines, aminoglycosides, chloramphenicol and trimethoprim (Carvalho et al., 2012). Though a variety of antibiotics proved useful to treat *A. hydrophila* infections, multiple antibiotic resistances are common among *A. hydrophila* (Orozova et al., 2008). Many strains of *A. hydrophila* are known to harbour mobile elements that encode antibiotic resistance and can be transferred among themselves or to other bacterial species to establish multiple antibiotic resistances (Al-Fatlawy and Al-Hadrawy, 2014). Janda and Abbott, (2010) reviewed that *Aeromonas* increased resistance rates against penicillins and first generation cephalosporins are associated with the production of chromosomally encoded beta-lactamases (Janda and Abbott, 2010).

Antibiotic resistant among *Aeromonas* isolates from aquatic environments has been reported to be principally plasmid mediated (Ghenghesh et al., 2001; USEPA, 2006). This is because resistance among *Aeromonas* is usually due to the horizontal transfer of mobile genetic elements such as plasmids and class 1 integrons. In addition, *Aeromonas* spp. usually demonstrate resistance to most β-lactams antibiotics due to the production of multiple inducible chromosomally encoded β-lactamases. (Ghenghesh et al., 2008; Orozova et al., 2008). This is of health concern because β-lactam antibiotics are the most widely used antibiotics for the treatment of bacterial associated infections, however, even previous data on environmental isolates have shown an increase in resistance to such β-lactam antibiotics (Beceiro et al., 2013). Moreover, data in a study done by Orozova et al., (2008) demonstrated that β-Lactam agents should be avoided in the treatment of *Aeromonas spp.* infections (Orozova et al., 2008).

Increased incidence of antibiotic resistance is said to have originated from high risk sources of contamination where antibiotics are often used. Environmental contamination with antibiotics also contributes to the spread and increase of antibiotic resistance genes amongst



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bacteria within the same aquatic environment (Huddleston et al., 2006). Several studies also confirmed that increased incidence of resistance in the water environment is due to misuse of antibiotics in human health, veterinary, aquaculture, agriculture and household products (Adesoji et al., 2014). The ease of which antibiotics can be obtained and inappropriate use are blamed for incidence of high resistance in developing countries (Ghenghesh et al., 2008).

Several studies have shown that motile *Aeromonas* species are generally resistant to penicillin, ampicillin, carbenicillin and ticarcillin. Susceptibility of isolated *Aeromonas* species in several studies done in developing countries demonstrated 100% resistant to ampicillin. They also showed resistance to cephaloridine, tetracycline, erythromycin, trimethoprim, and amoxicillin. Most studies on the other hand were susceptible to ceftriaxone, ciprofloxacin, chloramphenicol, gentamicin, kanamycin, nalidixic acid and trimethoprim-sulphamethoxazole (Ghenghesh et al., 2001; Nashnoush et al., 2009; Al-Sulami et al, 2014; Mulamattathil et al., 2014).

## 2.9. Identification and testing techniques for microbiological analyses of isolates

### 2.9.1. Analytical Profile Index (API) 20E biochemical system:

The API 20E system is a commercial kit that consists of 20 microtubules containing dehydrated substrate. It is used to identify Gram-negative bacteria based on their biochemical characteristics. A bacterial suspension is used to inoculate each cupule or well. Some of the wells change colours due to pH differences or metabolism whereas others produce end products that have to be identified with added reagents after incubation period of 24 hrs at 37°C. Each reaction is given a plus or minus sequence which is then converted into a numerical code. This numerical code provides a 7 digit profile for the organism. The profile number is entered into a computer identification model which then provides the identification of the organism (Abbott et al., 2003). Table 2.1 describe the 20 key biochemical test substrate included in API 20E kit and the expected results from commonly reported human pathogenic *Aeromonas* strains and one rarely reported species.



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**Table 2.1:** Key biochemical characteristics for identification of commonly reported *Aeromonas* spp. using API 20E biochemical technique.

| Test Substrate | Reaction tested | Commonly reported *Aeromonas* spp. | | | rarely reported spp. |
|---|---|---|---|---|---|
| | | *A. hdrophila* | *A. caviae* | *A. veronii bv. sobria* | *A.media* |
| ONPG (o-Nitro phenyl Galactoside ) | beta-galactosidase | + | + | + | + |
| ADH (arginine) | arginine dihydrolase | + | + | + | + |
| LDC (lysine) | lysine decarboxylase | + | - | + | - |
| ODC (ornithine) | ornithine decarboxylase | - | - | - | - |
| CIT (citrate) | citrate utilization | [+] | [+] | [+] | [+] |
| H2S (Na thiosulfate) | H2S production | + | - | + | - |
| URE (urea) | urea hydrolysis | - | d | - | - |
| TDA (tryptophan) | deaminase | - | - | - | - |
| IND (tryptophan) | indole production | + | + | + | [+] |
| VP (Na pyruvate) | acetoin production | + | - | + | - |
| GEL (charcoal gelatin) | gelatinase hydrolysis | + | [+] | + | [+] |
| GLU (glucose) | fermentation | + | - | + | - |
| MAN (mannitol) | fermentation | + | + | + | + |
| INO (inositol) | fermentation | - | - | - | - |
| SOR (sorbitol) | fermentation | - | d | - | - |
| RHA (rhamnose) | fermentation | d | - | - | - |
| SAC (sucrose) | fermentation | + | + | + | + |
| MEL (melibiose) | fermentation | - | d | d | - |
| AMY (amygdalin) | fermentation | [+] | [+] | - | - |
| ARA (arabinose) | fermentation | [+] | + | - | + |

+: 90% or greater positive; - :90% or greater negative; [+]: 50-89% of strains are positive; and d: only 4-49% of strains are positive.

Citations: (Abbott et al., 2003; Janda and Abbott, 2010; Igbinosa et al., 2012).

2.9.2. Haemolysin production:

Haemolysin production is usually tested on blood agar to test the enzymatic ability of bacteria to break down red blood cells by releasing haemolysins. Haemolysins are extracellular toxic proteins which possess certain pathogenic potential that cause lysis of red blood cells by damaging their cell membrane in certain bacterial Gram-negative and Gram positive species (Singh and Sanyal, 1992a,b). Furthermore, haemolysins are known diverse group of multifunctional enzymes that play a fundamental role in *A. hydrophila* pathogenesis (Rasmussen-Ivey et al., 2016).



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Haemolysis is classified in to alpha, beta and gamma based on the haemolytic activity of the toxins. Alpha haemolysis involves partial break down of the haemoglobin which is seen is observed as green discoloration forming on the agar around the streaked colony area. Beta haemolysis involves the complete breakdown of haemoglobin and is observed by a clear zone around the area of inoculation on blood agar. Gamma haemolysis represents no haemolysis. It appears brownish which is the normal reaction displayed by blood at 37°C in the presence of carbon dioxide (Santos et al., 1999). Haemolytic positive bacteria together with other virulence factors in bacterial species determine whether the bacterial isolates are potential pathogenic or not.

Most *Aeromonas* species possess the haemolytic activity to lyse the red blood cells when grown on a suitable blood agar. For this reason, the production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in *Aeromonas spp.* (Janda and Abbott, 2010). Furthermore, the production of particularly β-hemolysin toxin in motile *Aeromonas* isolates has been reported as one of the virulence factor of *Aeromonas* species (Majeed and MacRae, 1993; Erova et al., 2007). Since β-hemolysin, belongs to a larger group of poreforming bacterial cytolysins and it usually causes leakage of the cytoplasmic contents from target cells through disruption of the normal integrity of the cell membrane (Abrami et al., 1998).

2.9.3. 16S rRNA gene sequencing:

The rRNA gene consists of the most conserved DNA in bacterial cells (Clarridge, 2004). For this reason, a 16S rRNA gene sequence has been widely used in the last decade due to its presence in almost all bacteria. Recent literature showed that 16S rDNA sequencing play an important role in the accurate identification of bacterial isolates (Janda and Abbott, 2007). Moreover, 16S rDNA sequencing is particularly useful in identifying unusual bacteria that are difficult to identify by conventional methods or isolates observed with "unacceptable" biochemical profile, providing genus identification to species level (Woo et al., 2008).

At present, 16S rRNA bacterial gene sequencing is generally accepted as a stable and specific marker for bacterial identification purposes (Marchandin et al., 2003), since this gene is present in all prokaryotes. In general, the technique of 16S rRNA PCR is used to amplify ribosomal DNA, with the use of designed universal primers complementary to conserved regions in order to amplify the region from any bacteria. In completion of the amplification process, the amplified region is purified and then subjected to cycle sequencing PCR after



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which it is finally subjected to the 16S rRNA gene sequencing determination. The sequencing results are then usually analysed or identified using a BLAST homology search with isolates gene sequences deposited in the GenBank database (Clarridge, 2004).

Therefore, sequencing is generally regarded as a valuable tool when studying prevalence of bacteria as it provides information on genetic differences between different strains of the same organisms. Furthermore, it is able to identify the differences that cannot be detected with phenotypic or biochemical methods (Janda and Abbott, 2007; Woo et al, 2008). Literature further reported that one more interesting thing about sequencing when studying biofilms bacterial individuals' community is that it can also detect genetic differences and relatedness of the organisms within the biofilms in the same biofilm community (Eisen, 2007).

## 2.10. Summary of the literature review

Over the past few years a vast number of papers and reviews have been published on *Aeromonas* as an emerging human pathogen. Day by day *Aeromonas* species, particularly *A. caviae*, *A. hydrophila*, and *A. veronii* are becoming more important due to their increasing association with numerous human diseases, such as gastrointestinal conditions and extra intestinal conditions in both immunocompromised and immune-competent individuals. *Aeromonas* infections usually follow as a result of ingestion of contaminated water or food and as a result of trauma in aquatic environment. *Aeromonas* is currently included in the *Contaminant Candidate List by WHO* and the *US Environmental Protection Agency of potential waterborne pathogens* (USEPA, 2006; Igbinosa et al., 2012).

*Aeromonas* species are found worldwide in surface water, ground water, non-chlorinated drinking water, chlorinated drinking water, and bottled mineral water (Ghenghesh et al., 2008). The findings in a study done by Parker and Shaw, (2011) provide evidence of successful colonization and subsequent infection of a number of *Aeromonas* species after transmission from water to humans. Exposure to water contaminated with *Aeromonas* spp. through ingestion or skin contact can lead to human disease in both immune-competent and immunocompromised individuals with severe infections to immunocompromised individuals (Parker and Shaw, 2011).

Recently *Aeromonas* species are have been considered as enteric pathogens of serious public health concern as they possess a number of virulence factors that are believed to play a role



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in human infections, such as in gastroenteritis, wound infections, septicaemia, and skin diseases. They are also recognized as putatively emerging enteric pathogens, which are capable to grow in water distribution systems, where they may be resistant to chlorination. In developing countries, potentially pathogenic *Aeromonas* species are very common in drinking water and in different types of foods, particularly seafood (Igbinosa et al., 2012). Currently an increasing incidence of antibiotic resistance among emerging *Aeromonas spp.* isolated from aquaculture environments which are generally opportunistic human pathogens has been observed worldwide (Orozova et al., 2008).

*Aeromonas* species are also considered as one of the biofilm growing bacteria. Literature has stated that biofilm growing bacteria may be responsible for human chronic infections because of their ability to develop increased resistance to antibiotics (Igbinosa et al., 2012). This is of public health concern as many household members in rural areas of South Africa utilizes storage containers to store their drinking water which have been showed to encourage the formation of biofilm over a period of storage of the drinking water (Momba and Kaleni, 2002; Levy et al., 2008). This highlight the importance of regularly monitoring *Aeromonas* species in household stored drinking water. Not many studies in rural areas of the Limpopo province in South Africa has been done on the prevalence and antibiotic resistant patterns of *Aeromonas* bacteria in drinking water. Therefore this study focused on investigation of prevalence of *Aeromonas* species, their antibiotic resistance patterns and their virulence capabilities from drinking water collected from households' water storage containers in rural areas of the Limpopo province in South Africa.



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# Chapter 3
# METHODOLOGY

A summary of study design is given in Figure 3.1.

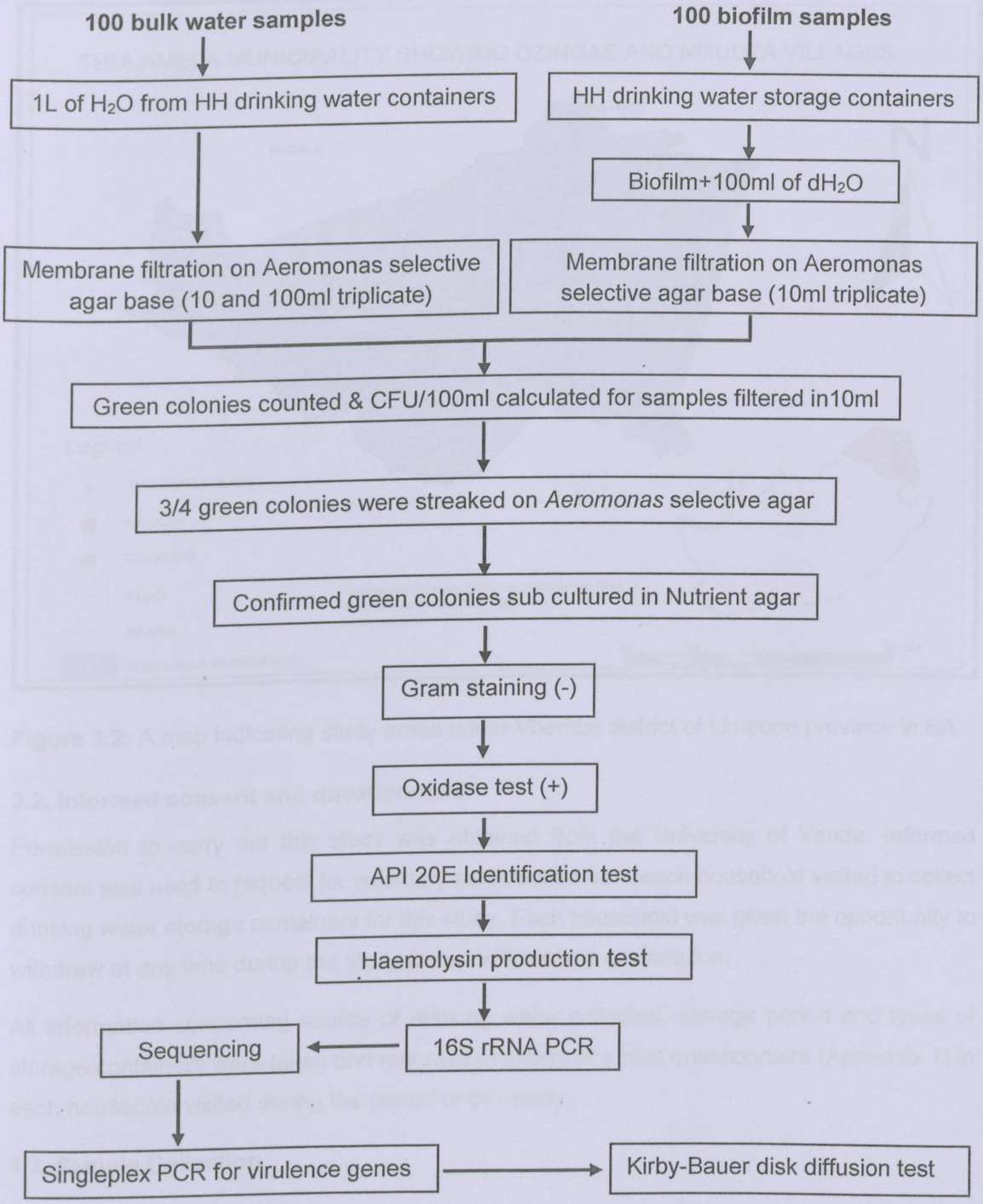



**Figure 3.1:** A flowchart summary of study design or methodology



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### 3.1. Study area

This study was conducted in the Ngudza and Dzingahe rural villages in Vhembe district of South Africa (Figure 3.2).

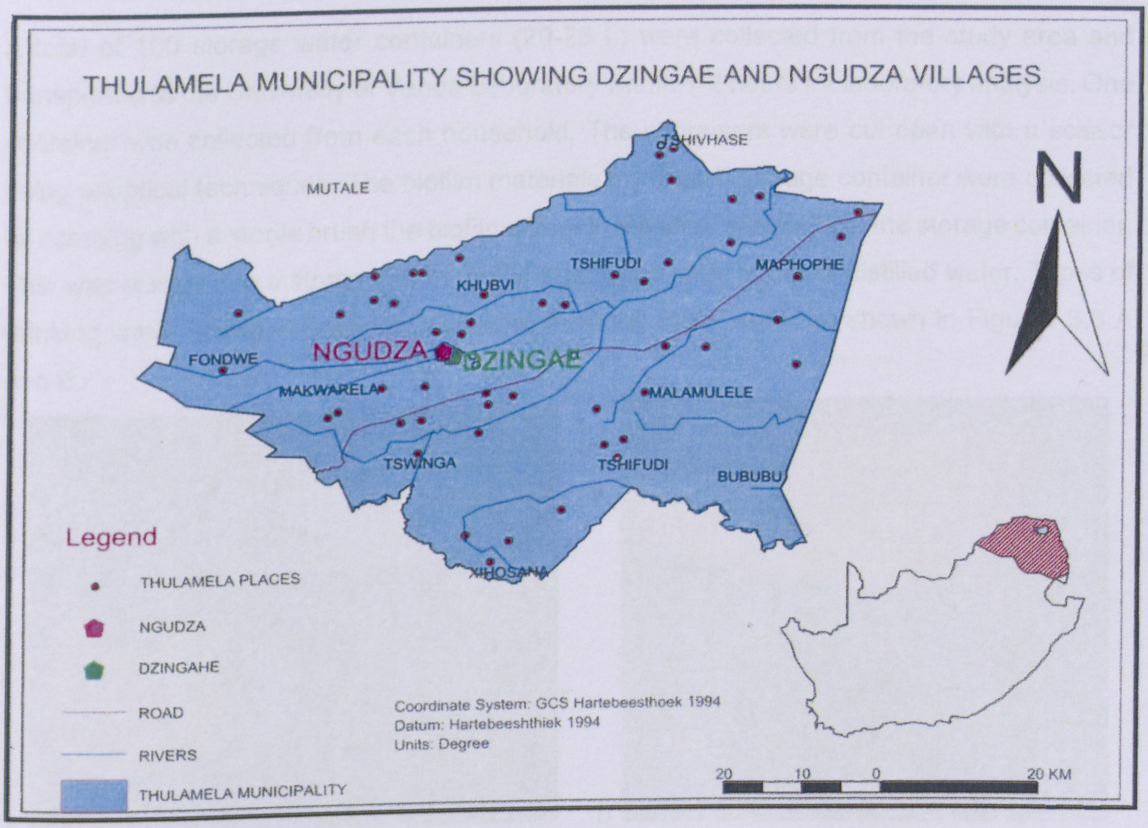



**Figure 3.2:** A map indicating study areas under Vhembe district of Limpopo province in SA

### 3.2. Informed consent and questionnaire

Permission to carry out this study was obtained from the University of Venda. Informed consent was used to request for voluntary participation from each household visited to collect drinking water storage containers for this study. Each household was given the opportunity to withdraw at any time during the study period without any explanation.

All information concerning source of drinking water collected, storage period and types of storage containers were taken and recorded in a form of a pilot questionnaire (Appendix 1) in each household visited during the period of this study.

### 3.3. Sample Collection

One liter water samples were collected from household water storage containers (20 or 25 L) of 100 households. A questionnaire was used during sample collection to obtain information



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concerning storage period of water within households containers before consumption, water source, availability of water and type of storage containers used. The samples were placed in a cooler box and transported to the University of Venda Laboratory within two hours for Lab analysis.

A total of 100 storage water containers (20-25 L) were collected from the study area and transported to the University of Venda Laboratory within 1-2 hours for laboratory analysis. One container was collected from each household. The containers were cut open with a scissor using aseptical techniques. The biofilm materials from each storage container were collected by scraping with a sterile brush the biofilms from in the inner sidewalls of the storage container. This was placed into a sterile 250 ml bottle and mixed with100 ml of distilled water. Types of drinking water storage containers collected from the study area are shown in Figures 3.3 A and B.

A  B 

**Figure 3.3:** (A) Types of drinking storage containers (20-25 L) used at the study area. (B) A representative of a cut open drinking water storage container with biofilms.

### 3.4. Isolation of *Aeromonas* bacteria from water and biofilm samples

3.4.1. Isolation of presumptive *Aeromonas* bacterial colonies from bulk water samples

Volumes of 100 ml and 10 ml from each water sample was separately filtered through 0.45 µm pore sized filters in triplicate using the membrane filtration technique. The membranes were aseptically placed on prepared *Aeromonas* agar base (Oxoid) selective media (Biolab, Merck, SA) and incubated at 37°C for 24 hours. After incubation, green colonies were taken as presumptive *Aeromonas* spp., counted and the CFU/100 ml calculated. Typical colonies were selected from membranes and purified for further analysis.



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3.4.2. Isolation of presumptive *Aeromonas* bacterial colonies from biofilm samples

Ten milliliters of each biofilm sample was filtered through 0.45 µm pore sized filters. This was conducted in triplicate. The membranes were aseptically placed on prepared *Aeromonas* agar base (Oxoid) selective media (Biolab, Merck, SA) and the plates were incubated at 37°C for 24 hours. After the incubation period, green colonies were taken as presumptive *Aeromonas* spp. All presumptive *Aeromonas* colonies were counted CFU/100 ml calculated. Green colonies were then selected from membranes and purified for further identification.

**3.5. Purification of colonies and Gram staining test**

All presumptive positive colonies were purified by sub-culturing on *Aeromonas* selective agar and incubating for 24 hours at 37°C. Purified cultures were then further sub-cultured on Nutrient Agar and incubated for 24 hours at 37°C using the streak plate method. Single colonies for each sample were subjected to Gram staining. All the Gram-negative isolates were streaked on to Nutrient Agar to prepare pure cultures that were used for further identification which includes biochemical identification tests (oxidase and API 20E), PCR assays and antibiotic resistance tests. The prepared cultures were stored in a refrigerator until used for further analysis.

**3.6. Biochemical Identification tests**

3.6.1. Oxidase Test

The oxidase test was performed using the test oxidase reagent (PL.390) from Mast Diagnostics (Nesto, Wirral, United Kingdom) according to the manufacturer's protocol. Briefly: A well-isolated pure colony was placed on a filter paper using a sterile wire loop. A drop of test oxidase reagent was added to it and mixed immediately. After 30 seconds, the filter paper was observed for a colour change with the oxidase positive isolates producing a purple colour. All the colonies that changed to purple colour were taken as presumptive *Aeromonas* isolates and used for further identification. All the colonies with no colour change were considered as oxidase negative and therefore discarded.



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3.6.2. Analytical Profile Index (API) 20E Test

The API 20E test was performed according to the manufacturer's protocol (BioMerieux) as follows: Firstly a tray and lid was prepared and approximately 5ml of distilled water was sprinkled into the bottom of the tray for each bacterial isolate. An API 20E test strip was placed into the bottom of the tray and the microtubes of the kit were filled with bacterial suspension of the presumptive positive isolates. The incubation box was then closed and incubated at 37°C for 24 hrs. After incubation, reactions were developed by adding 1 drop of specific reagents to specific tubes in the tray as specified by the manufacturer. After 10 minutes the reactions were read by referring to the table supplied by the manufacture and results were recorded as a 7 digits code. All the Gram negative, oxidase positive isolates that were considered as presumptive *Aeromonas* were then identified by interpreting the 7 digits code to species level using API software. Isolates that were identified as *Aeromonas* were recorded and used for further analysis and those that were identified as other bacterial species were only recorded, but were not further analysed.

3.6.3. Haemolysin production on Blood Agar

Commercially prepared Blood Agar (Biolab, Merck, SA) supplemented with 5% sheep blood was used to test for haemolysin production. Haemolysis was determined by streaking the Gram negative, oxidase positive isolates on purchased blood agar plates. The plates were incubated at 37°C for 24 hours. After incubation the medium was inspected for alpha- or beta-haemolysis. For the medium that was discoloured or darkened after incubation, the organism was considered to indicate alpha-haemolysis. If the medium developed a clear halo after incubation, the organism was considered to indicate beta-haemolysis. On the medium where no colour change was observed, the organism was considered to indicate gamma haemolysis.

**3.7. Molecular Identification for further analysis of *Aeromonas* isolates**

3.7.1. Extraction of DNA

The DNA isolation method according to Carstens et al. (2013) was used. Briefly into a 1.5 ml Eppendorf tube containing 20 µl broth, a bacterial colony was added and mixed. The tubes were centrifuged at maximum speed for 1 minute. All the supernatant was removed from centrifuged tubes using a micropipette and 15 µl of nuclease free PCR water (Fermentas Life Science, US) was added into the tubes and mixed with the pellet. The tubes were then



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vortexed to make sure that the added water is mixed with the pellet containing bacterial cells. The tubes were subsequently sealed with plastic sealers to prevent them from popping open during a microwave exposure step. These were microwaved for 2 minutes at maximum power. Afterwards, the tubes were centrifuged at maximum speed for 90 seconds. After centrifugation the tubes were immediately placed on ice. A volume of 1 µl supernatant was used as DNA for each PCR reaction.

### 3.7.2. 16S rRNA gene sequencing for confirmation of presumptive positive API 20E isolates

The *Aeromonas* species identities obtained with the API 20E biochemical identification technique were confirmed using 16S rRNA gene sequencing. Each 16S PCR reaction was performed in a total volume of 20 µl containing 2 µl of bacterial DNA template (50-100 ng/µl), 6.4 µl of RNase/DNase free $H_2O$ (Fermentas Life Science, US), 10 µl of 2x PCR Master Mix (0.05 U/µL Taq DNA polymerase in reaction buffer, 0.4mM of each dNTP and 4 mM $MgCl_2$) (Fermentas Life Science, US), and 0.4 µM of 27F and 1492R primers set (Table 3.1; Inqaba Biotech, SA). The cycling conditions for amplification of 16S rDNA gene from isolates were set as follows: Initial denaturation at 94°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, annealing at 54°C for 30 seconds and at 72°C for 1 minute. This was followed by final extension at 72°C for 5 minutes.

The confirmation of 16S PCR products was done by electrophoresis on 1% (w/v) agarose gel (PeQlab, Germany) using 1 x TAE buffer [20mM Acetic acid (Merck, US), 40mM Tris (Sigma Aldrich, US) and 1mM EDTA (Merck, US) at pH 8.0] for 45 minutes at 80V. A mixture of 3µl PCR product and 3 µl of 6x Orange Loading dye (Fermentas Life Science, US) containing GelRed dye was loaded into each well of the gel. Additionally, a 1 kb molecular marker (O'GeneRuler, Fermentas Life Science, US) was loaded into the gel in order to confirm the fragment sizes of the amplified rDNA PCR product. A Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software was used to capture the gel images (Bezuidenhout *et al.*, 2006).



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**Table 3.1**: Primers set used for 16S rRNA PCR reactions

| Target Gene | Primers Sequence (5'-3') | Size (bp) | Reference |
|---|---|---|---|
| 16S rRNA | **27F:** 5'-AGAGTTTGATCMTGGCTCAG-3'<br>**1492R:** 5'-CGGTTACCTTGTTACGACTT-3' | 1465 | Jiang et al., 2006 |

### 3.8. Singleplex PCR for detection of virulence genes in isolates

The same DNA isolated previously were used during this step. Each PCR reaction was performed in a total volume of 25 µl containing 1 µl of bacterial DNA template (50-100 ng/ µl), RNase/DNase free $H_2O$ (Fermentas Life Science, US), 2x Dream Taq PCR Master Mix (Thermo Fisher Scientific, SA) (0.05 U/µL Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM $MgCl_2$), 0.2 µM of primers *asa1* and *gelE* as well as 0.4 µM of primers cylA and hyl (Inqaba Biotech, SA). Isolates were amplified using the following cycling conditions; Initial denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, annealing at 56°C for 30 seconds and at 72°C for 1 minute. This was followed by a final extension step of 72°C for 10 minutes.

Electrophoresis was conducted in a horizontal Pharmacia Biotech equipment system (model Hoefer HE 99X; Amersham Pharmacia Biotech, Sweden). The PCR products were separated by electrophoresis on 1% (w/v) agarose gel (PeQlab, Germany). This was conducted for 90 minutes at 60V using 1x TAE buffer (40 mM Tris, 1 mM EDTA, and 20 mM glacial acetic acid, pH 8.0). A mixture of 10 µl PCR product and 3 µl of 6x Orange Loading dye (Fermentas Life Science, US) mixed with gel-red was loaded into each well of the gel. Additionally, a 100 bp or 1 kb DNA molecular weight marker (Fermentas, USA) was loaded into the gel in order to confirm the targeted gene fragment sizes. After running gel electrophoresis, a Gene Genius Bio imaging system (Syngene, Synoptics, UK) was used to view and capture the image using GeneSnap (version 3.07.01) software (Syngene, Synoptics, UK).



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**Table 3.2:** Oligonucleotide primers set used for PCR reactions

| Virulence factor | Target Gene | Oligonucleotide Primers Sequence (5'-3') | Size (bp) | Reference |
|---|---|---|---|---|
| Aggregation substance | *Asa1* | **ASA 11:** 5'-GCACGCTATTACGAACTATGA-3' **ASA 12:** 5'-TAAGAAAGAACATCACCACGA-3' | 375 | Vankerckhoven et al.,2004 |
| Cytolysin | *cylA* | **CYT I:** 5'-ACTCGGGGATTGATAGGC-3' **CYT II b:** 5'-GCTGCTAAAGCTGCGCTT-3' | 688 | Vankerckhoven et al.,2004 |
| Gelatinase | *gelE* | **GEL 11:** 5'-TATGACAATGCTTTTTGGGAT-3' **GEL 12:** 5'-AGATGCACCCGAAATAATATA-3' | 213 | Vankerckhoven et al.,2004 |
| Hyaluronidase | *hyl* | **HYL n1:** ACAGAAGAGCTGCAGGAAATG-3' **HYL n2:** 5'-GACTGACGTCCAAGTTTCCAA-3' | 276 | Vankerckhoven et al.,2004 |

### 3.9. Antibiotic Resistance testing of the isolates

The following antibiotics and their concentrations were used: ampicillin (AP) 10 µg, amoxicillin (A) 10 µg, cephalothin (KF) 30 µg, ciprofloxacin (CIP) 5 µg, chloramphenicol (C) 10µg, erythromycin (E) 15 µg, kanamycin (K) 30 µg, oxytetracycline (OT) 30 µg, streptomycin (S) 10 µg, and trimethoprim (TM) 2.5 µg. These antibiotics were chosen because they are either commonly used in human medicine as well as in veterinary practice and also because previous studies have reported microbial resistance to them.

Antibiotic resistance tests were performed using the Kirby-Bauer disk diffusion method for each sample isolate as follows: Four to five pure colonies were picked representing each sample isolates. These were transferred in to 5 ml of sterile distilled water to prepare bacterial suspension which is usually similar to the McFarland standard. Aliquot of 100 µl from each bacterial suspension was spread-plated on Mueller-Hinton agar plate. Antibiotic discs were then placed on the plates using sterile needles and the plates were incubated at 37°C for 24



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hours. After incubation, the antibiotic inhibition zone diameters were measured. Results obtained were interpreted to classify isolates as being resistant, intermediate resistant, or susceptible to a particular antibiotic using standard reference values according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2007), previously known as the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS 2003).

### 3.10. Statistical analysis

The data was statistically analysed using Microsoft Excel 2010 and the IBM SPSS statistical Software (v.22).

A Student's t test was used to evaluate any statistically significant differences between the CFU mean values of the bulk water and biofilm isolates. The significant different of the antibiotic resistance levels between bulk water and biofilm isolates from tap and river water sources were also determined. A P-value of $< 0.05$ was considered to be the level of statistical significance.



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# Chapter 4
# RESULTS AND DISCUSSION

This study was aimed at determination of the prevalence and antibiotic resistance patterns of *Aeromonas* spp. from drinking water in storage containers from rural households in the Vhembe district of South Africa. The results are shown and discussed in detail in the following sections:

## 4.1. Questionnaire data

According to the questionnaire data taken, all members of the households visited in the study area used plastic 20 or 25L storage containers to collect and store water for drinking. This water was also used for other domestic purposes such as food preparation, bathing, and washing. Majority of the household dwellers stored their water for drinking for a period of 1 day to 1 week. Twenty percent stored water for up to 3 weeks and 4% indicated that they may store the water for up to 2 months before use, depending on the water availability at the public stand pipe within the community.

**Table 4.1:** A summary of questionnaire data taken and documented during sampling of bulk water and biofilm samples from 100 household drinking water storage containers.

| ***Characteristics of collected households' drinking water*** | ***Questionnaire data taken during samples collection*** | ***No. of households*** |
|---|---|---|
| *Source of water sampled* | Tap water | 69 |
| | River water | 30 |
| | Borehole | 1 |
| *Types of storage containers* | Plastic 20 or 25L containers | 100 |
| *Storage Period of water before use* | 1 to 5 days | 41 |
| | 1 week | 35 |
| | 2 weeks | 14 |
| | 3 weeks | 6 |
| | 1-2 months | 4 |
| *Alternative water source (If water is unavailable at the main supply source)* | River water | 56 |
| | Borehole and in house pipe | 18 |
| | Tank and enough storage containers | 16 |
| | Nearest village tap | 10 |
| *Treatment of river water before use (where applicable)* | Jik/Bleach | 6 |
| | Salt | 2 |
| | Boil for drinking | 9 |
| | Nothing | 56 |
| | Sachet from clinic if available | 1 |
| *Type of transport used from public stand pipe to the household* | Travel/walk | 65 |
| | Hire a car | 15 |
| | Wheelbarrow | 4 |
| | In house pipe | 16 |



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| | | |
|---|---|---|
| *Immunocompromised household members* | Ranges from 1-4 people per household<br>None | 39<br>61 |

Most of the households (74/100) used river water as an alternative water source during the period in which water is unavailable at the public stand pipes. In this case 76% used the river water without implementation of any treatment procedure (Table 4.1). The use of untreated water for drinking and other domestic purposes might expose the consumers to potential pathogenic bacteria including *Aeromonas* species since they occur naturally in untreated water sources (WHO, 2011). Furthermore, immunocompromised individuals who reside in visited households (40% of the households; Table 4.1) relied on such water for drinking which then posed a significant health risk, since ingestion of *Aeromonas* and other pathogens or opportunistic pathogens contaminated water may result in severe infections in immunocompromised individuals (Parker and Shaw, 2011; Igbinosa et al., 2012).

## 4.2. Colony-forming unit (CFU) of presumptive positive *Aeromonas* spp. and their growth characteristics

All bulk water and biofilm samples filtered through membrane filtration technique in this study displayed small and large round dark green colonies on *Aeromonas* agar base selective media at 37°C for 24 hours. These were regarded as presumptive *Aeromonas* isolates. Dark green colonies were counted and for each sample filtered in 10 ml were calculated to 100 ml and purified for further analysis. Representative petri-dishes indicating presumptive *Aeromonas* isolates are shown in Figure 4.1 (A) and (B). The presumptive positive dark green colonies were counted and recorded as indicated in Table 4.2.

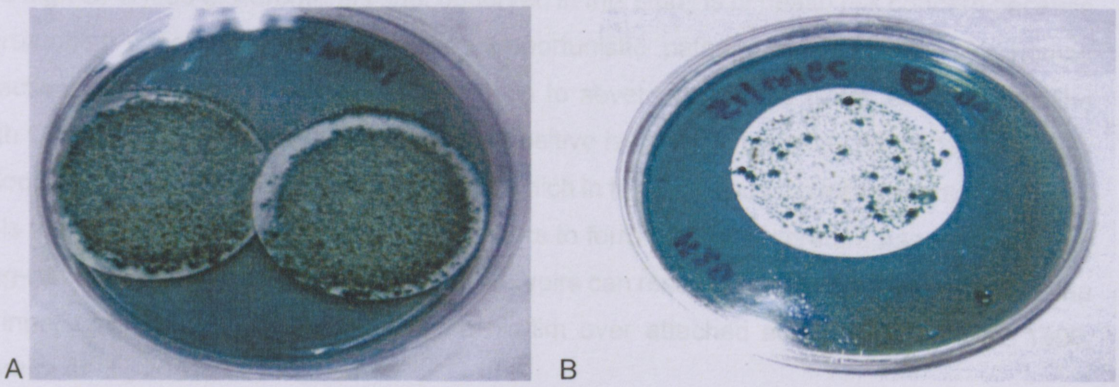


**Figure 4.1:** A representative of presumptive *Aeromonas* on agar indicating the dark green colonies. (A) represents 100 ml (B) 10 ml of water that was filtered through 0.45 µm filters.



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The minimum CFU count of 8 x 10 and the maximum CFU count of greater than $3.0 \times 10^2$ with the mean value of $1.52 \times 10^2$ CFU per 100ml of bulk water samples analysed was observed. The *Aeromonas* presumptive positive isolates for biofilm samples displayed a minimum CFU count of $1.0 \times 10^1$ and the maximum count of greater than $3.0 \times 10^2$ with the mean value of $2.40 \times 10^2$ per 100ml. There is no statistical significant difference in the CFU mean values (P=0.760) observed between bulk water and biofilm isolates (Table 4.2). The CFU mean value of *Aeromonas* isolates was slightly higher in biofilm isolates when compared with the bulk water isolates. At present there are no known documented regulatory standards for the acceptable number of *Aeromonas* in drinking water in South Africa. However, according to the EPA, the number of *Aeromonas* species in treated drinking water is typically below 10 CFU per 1ml (Percival et al., 2004; USEPA, 2006). Furthermore, the World Health Organization has reported the isolation ratio of 1.0-27% *A. hydrophila* in drinking water (WHO, 2001). However, most CFU mean values data for presumptive positive *Aeromonas* isolates in the present study exceeded the EPA recommended standard limit and the WHO's isolation ratio water quality standards.

**Table 4.2:** CFU of presumptive positive *Aeromonas* spp. isolates from bulk water and biofilm samples per 100ml

| ***Aeromonas* presumptive positive isolates** | **CFU/100 ml of samples analysed** | |
|---|---|---|
| | **Bulk water isolates** | **Biofilm isolates** |
| **Minimum count** | 8 x 10 | $1.0 \times 10^1$ |
| **Maximum count** | $\geq 3.0 \times 10^2$ | $\geq 3.0 \times 10^2$ |
| **Mean** | $1.52 \times 10^2$ | $2.40 \times 10^2$ |

The high CFU load of *Aeromonas* spp. observed in this study is of health risk concern because *Aeromonas* species are recognized as opportunistic pathogens responsible for human infections ranging from gastrointestinal illness to severe conditions (Riley et al, 2011). The high CFU count of *Aeromonas* presumptive positive isolates in this study might be the results of long storage periods of the drinking water, which in turn influence the attachment of bacterial cells to the inner walls of the storage containers to form biofilms. Literature has indicated that long-term water storage in finished water reservoirs can result in waterborne bacteria attaching to inner walls, growing and spreading a biofilm over attached surfaces (Geldreich, 1996; Momba et al., 2000).



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A study done in Netherlands frequently observed *Aeromonas* spp. in drinking water in numbers varying between 1 and $1.0x10^4$ CFU/100 ml (Havelaar et al., 1990). Data reported from a study done in Italy displayed *Aeromonas* spp. in water samples in counts ranging from 26 to 1 609 CFU/250 ml (Massa et al., 2001). In a study conducted in northeastern Italy (the Dolomites) on drinking water supplies *Aeromonas* levels range from 1 to 240 CFU/100 ml was observed (Legnani et al., 1998). Furthermore, a study conducted in Sweden reported tap water samples that contained presumptive *Aeromonas* spp. that exceeded 10 CFU/100 ml with a maximum count of 750 CFU/100 ml. Raw water had counts exceeding 1 000 CFU/100 ml (Kuhn et al., 1997). These are examples where results were similar to that of the present study. However, some studies have reported much lower levels of presumptive positive *Aeromonas* spp. in water. Figueras et al., (2005) found only few CFU per 100 ml in drinking water in Spain. Moreover, a study done in Lebanon by Tokajian and Hashwa (2004) observed a range of 3 to 43 CFU per 100 ml of *Aeromonas* species in drinking water.

*Aeromonas* is now considered as an additional indicator of contamination in several countries due to its high CFU/100ml counts frequently observed in drinking water (WHO, 2008). Species of the genus *Aeromonas* were isolated more frequently than *E.coli* from environmental samples, although both *Aeromonas hydrophila* and *E. coli* have the same survival capability. This highlighted the fact that the prevalence of *Aeromonas* species in any drinking water sources could pose for a potential public health risk (Pandove et al., 2013).

### 4.3. Identification of presumptive positive isolates with biochemical identification tests

#### 4.3.1. Gram staining and Oxidase test

Gram staining and oxidase tests were performed on all the purified presumptive positive bulk water and biofilm isolates. All presumptive positive bulk water and biofilm isolates tested in this study were Gram negative. All Gram-negative isolates were then tested for the production of cytochrome oxidase enzyme. Out of 100 Gram-negative *Aeromonas* presumptive positive bulk water samples and biofilm samples analysed in the present study, 84% and 83% isolates respectively tested oxidase positive. These oxidase positive isolates were then subjected to the API 20E biochemical identification technique.

#### 4.3.2. Identification of isolates using API 20E identification technique

Further identification of *Aeromonas* presumptive positive isolates based on their biochemical characteristics was determined through the use of the API 20E biochemical identification



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technique (Chapter 3: Section 3.5.2) and the results are illustrated in Table 4.3. According to the API 20E identification system, *Aeromonas* identification to species level was all similar representing only the possibility of either one of the three species. Thus, API 20E biochemical identification system was not specific in terms of identifying only one species per isolate; it only identified isolates with the possibility of either one of the species *Aeromonas hydrophila/ caviae/sobria 1 and or 2* within the genus *Aeromonas.* However, not all *Aeromonas* presumptive positive isolates were identified as members of the genus *Aeromonas*. A number of bacterial species of which the majority belong to the family Enterobacteriaceae were also identified from the presumptive positive isolates. These bacterial species included *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Raoultella ornithinolytica*, *Serratia odorifera 1*, *Serratia marcescens*, *Serratia liquefaciens*, *Cronobacter spp*, *Vibrio cholerae*, and *Vibrio fluvialis*.

4.3.2.1 Prevalence of *Aeromonas* spp. from bulk water isolates in accordance with API 20E biochemical identification system

Out of the 84% of presumptive positive bulk water isolates from households drinking water analysed in this study, 73 (44%) of which 46 (28%) were from river water storage container and 27 (16%) were from tap storage containers and were identified as *Aeromonas* species using the API 20E biochemical identification technique. The prevalence of 44% *Aeromonas* species from households' drinking water could be due to the long storage period of such water in storage water containers as water is not occasionally available from the community taps in the study area. In addition, formation of biofilm due to reduced chlorine levels in storage drinking water containers increases the opportunity for regrowth and multiplication of *Aeromonas* bacteria in drinking water and as a result this degrades the microbiological quality of water. Furthermore, it has been reported that *Aeromonas* spp. recovered from biofilm are resistant to disinfection due to the protective effect of the extra polysaccharide substances (EPS) (Fernandez et al., 2000; September et al., 2007). Additional water contamination problems arise when the storage water containers are not regularly cleaned or closed tightly and therefore could be easily exposed to any environmental bacterial pathogen as reported in literature (Potgieter, 2007).

The high occurrence of *Aeromonas* species was observed from stored drinking water obtained from river or untreated source in accordance with the questionnaire taken in this study. This could be the results of *Aeromonas* collected from the river water source that has resulted in



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multiplication within the water storage containers. The high prevalence of these bacterial species indicated that water from river sources can serve as a source of contamination and this can expose users to associated infections. The results in this study are consistent with the literature in pointing out that the drinking of untreated water is the most likely manner to get *Aeromonas* infections (USEPA, 2006; Ghenghesh et al., 2008). Therefore the high prevalence of *Aeromonas* species in household drinking water obtained from river source present a health risk as the community dwellers depend of river water as one of the main alternative water supply during the period in which water is unavailable in community public standpipe.

There are limited local studies that have been documented concerning *Aeromonas* prevalence in household drinking water in local areas where this study was conducted, in rural areas of South Africa. *Aeromonas* was isolated in 12.0% and 23.8% of households' drinking water of HIV-positive individuals with diarrhoea and of HIV negative individuals with diarrhoea respectively (Obi et al., 2007). The study suggested that the high percentage of *Aeromonas* species isolation from household drinking water of HIV-positive individuals with diarrhoea in contrast with the low frequency of isolation from those without diarrhoea strongly associates the pathogens in diarrhoeal cases of HIV-positive individuals (Obi et al., 2007). Therefore the presence of *Aeromonas* spp. in household stored drinking water is a potential health risk to immunocompromised household inhabitants.

In studies elsewhere in Africa and the middle East *Aeromonas* isolates was reported in 35.7% of drinking water samples in Iraq (Al-Sulami et al., 2014); 45% drinking water from the El-Minia governorate in Egypt (Mahdi et al., 2008) and 73% of underground drinking water reservoirs samples analysed from Tripoli, Libya (Nashnoush et al., 2009). Similar to this study *Aeromonas* were also isolated from 36.5% of the samples of untreated sources or less protected raw drinking water sources which included wells, springs, fountains and mineral water in Brazil (Di Bari et al., 2007). *Aeromonas* was also isolated in 48.7% of untreated well water in Libya (Ghenghesh et al., 2001). In Iraq, *Aeromonas* spp. were isolated from raw water (river) and well waters at high percentage of 72.52%, and 35% respectively (Oleiwi et al., 2014). This is also in agreement with a study done in India, where 55% of *Aeromonas* isolates from environmental water samples was observed (Indra et al., 2016).

In contrast to the present study, a lower occurrence of *Aeromonas* spp. were observed in several studies done in different types of drinking water sources inclusive of the following studies. In Lecce, (Italy) Massa et al., (2001) reported *Aeromonas* in 25% of well water



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samples. Koksal et al., (2007) detected *Aeromonas* spp. prevalent in 6% of tap water samples and in 12% of domestic tank water samples in Istanbul, Turkey. In Spain, a study done by Pablos et al., (2009) reported that *Aeromonas* was present in 26.5% of drinking water samples analysed. In addition, 8.8% of drinking water distribution system samples were positive for *Aeromonas* spp. in Iraq (Oleiwi et al., 2014). In Brazil 21.9% of well water samples were reported positive for *Aeromonas* (Razzolini et al., 2008). In India the prevalence of *Aeromonas* spp. was as low as 18% (Didugu et al., 2015). Furthermore, *A. hydrophila* was never detected in the distributed bulk water in a study conducted in Indiana, USA on both bulk water and biofilm from water treatment plant distribution system (Chauret et al., 2001). The results from this study suggested sufficient chlorine levels on suspended bacterial species in water analysed.

4.3.2.2 Prevalence of *Aeromonas* from biofilm isolates in accordance with API 20E biochemical identification system.

Of the 83% presumptive *Aeromonas* spp. isolates identified by API 20E 45 (54%) were from biofilms. Twenty eight (34%) of these were from containers that stored river water and 17 (20%) were from containers that stored tap water, household river storage and household tap storage water respectively. The data in this present study thus emphasizes the role of biofilms in supporting the survival of *Aeromonas* species in drinking water. The data further suggest that the presence of biofilms within the storage containers provide favourable growth condition of *Aeromonas* species and as a result contribute to contamination of the household's drinking water.

The identification of *Aeromonas* strains from the biofilm isolates in this study is in agreement with another South African study that recovered *Aeromonas* spp. from biofilms from drinking water distribution system (September et al., 2007). Literature indicated that *Aeromonas hydrophila* species associated with biofilms would survive chlorine level which is sufficient to eradicate biofilm-associated *E. coli* (Desai and Desai, 2014).

The recovery of a number of *Aeromonas* strains from biofilm further presents the contamination of water when the biofilm is dislodged and this could be health risk particularly to immunocompromised individuals. *Aeromonas* spp. are considered as opportunistic bacterial pathogens that mainly affect immunocompromised individuals with more severe infections than in immune-competent individuals (Ghenghesh et al., 2008). In the present



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study immunocompromised individuals were present in the various households (Table 4.1). Some of them were proned to diarrhoea.

Bacterial species are capable of regrowth in drinking water distribution systems. However, studies on healthy individuals showed that very high infective doses of ($10^6$-$10^{10}$ cells) are needed for the development of infection (USEPA, 2002). This is however, not the case with immune compromised individuals. Care should thus be taken when drinking water needs of this group of individuals are considered and the result from the present study should be seriously considered for intervension programs.

### 4.4. Identification of isolates using 16S rRNA sequencing identification system

16S rRNA sequencing was used for the confirmation and comparison of API 20E identification results. Although a total of 118 bulk water and biofilm isolates were identified as *Aeromonas* spp. by biochemical tests performed, only 78 were selected based on different API 20E profiles and subjected to 16S RNA sequencing.

DNA was extracted according to the protocol described in Section 3.6.1 and 16S rRNA gene was amplified under the cycling conditions described in Section 3.6.2 using the primer sets indicated in Table 3.1. Figure 4.4 shows the expected DNA product size required for further sequencing analysis.

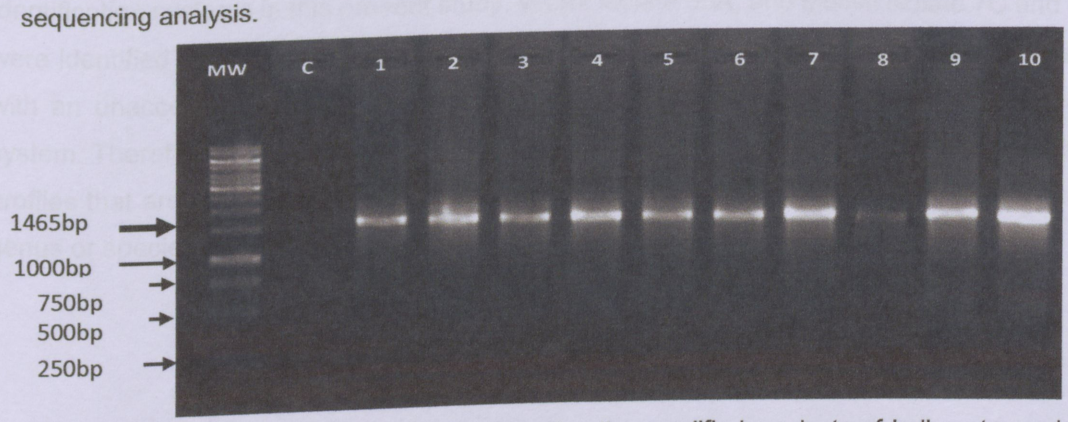



**Figure 4.2:** A 1% (w/v) agarose gel representing the amplified products of bulk water and biofilm pure isolates.

MW represents the 1kb molecular size marker (GeneRuler™ 1kb DNA ladder, ready to use, Fermentas Life Science, US). Lanes 1-5 contain bulk water samples and lanes 6-10 contain biofilm samples.



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The 16S RNA sequencing reaction was done as described in Sections 3.6.2 using the amplified DNA illustrated in Figure 4.4. GenBank was used to identify the sequences (http://www.ncbi.nlm.nih.gov/BLAST). The sequences used represented between ~200 to ~700 base pairs (Table 4.3). Out of 78 isolates sequenced, 30 were confirmed as *Aeromonas* species. The other 48 did not match identities obtained with the API 20E identification technique and also did not match any sequences of members of *Aeromonas* strains were not similarly identical with any members of *Aeromonas* strains stored within the Genbank sequence database. Most of the confirmed identities had E value of 0.0, thus indicating that those confirmed *Aeromonas* identities can be considered to be very close to the certain species obtained through Genbank blast.

16S rRNA sequencing data showed between 97 and 99% sequence similarity for 17 of the sequences. Lower sequence similarities that range from 71-96% was observed for 13 isolates. Some of the isolates that were identified as *Aeromonas* spp. by API 20E were not confirmed as *Aeromonas* spp. by 16S RNA, however were identified as opportunistic bacterial species as illustrated in Table 4.5. Furthermore, 16S rRNA sequencing technique successfully identified "unacceptable" or unusual phenotypic profiles observed with the API 20E identification systems in this present study. Water isolate 55A, and biofilm isolate 7C and 86C were identified as *Aeromonas* spp. by 16S rRNA sequencing, although they were observed with an unacceptable identification profile according to API 20E biochemical identification system. Therefore 16S rRNA sequencing technique is useful in identification of unacceptable profiles that are difficult to identify using API 20E biochemical identification technique to the genus or species level.



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**Table 4.3:** Bulk water and biofilm isolates confirmed as species of the Genus *Aeromonas* by 16S rRNA sequencing

| Isolates | API 20E Identification technique | 16S Sequencing ID | Nucleotides used | Similarity | Accession number | E value |
|---|---|---|---|---|---|---|
| **Bulk water:** | | | | | | |
| 17B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas media* | 90-530 | 99% | KC914093.1 | 0.0 |
| 55A | No Identification | *Aeromonas hydrophila* | 80-270 | 97% | KT361193.1 | 0.0 |
| 57B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 80-540 | 98% | KT624629.1 | 0.0 |
| 61B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 50-600 | 99% | KT379958.1 | 0.0 |
| 72B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 80-490 | 98% | KU570318.1 | 0.0 |
| 87A | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 10-300 | 83% | KC344850.1 | 0.0 |
| 92B | *Aeromonas hydrophila/caviae/ sobria 1* | *Aeromonas media* | 80-520 | 97% | KP813853.1 | 0.0 |
| 95B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 80-440 | 85% | KC793902.1 | 1E-103 |
| 97B | *Aeromonas hydrophila/caviae/ sobria 1* | *Aeromonas hydrophila* | 20-530 | 97% | KT379958.1 | 0.0 |
| 98B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas caviae* | 20-230 | 79% | KP120939.1 | 1E-29 |
| 99B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 30-530 | 99% | KT215607.1 | 0.0 |
| 100B | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 90-530 | 87% | KP813785.1 | 1E-159 |
| **Biofilm:** | | | | | | |
| 6C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 80-640 | 99% | KJ879242.1 | 0.0 |
| 7C | No Identification | *Aeromonas hydrophila* | 40-320 | 81% | KU1188292.1 | 1E-70 |
| 17C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas media* | 80-390 | 95% | KP813853.1 | 2E-140 |
| 19C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 80-270 | 71% | KU570297.1 | 1E-140 |
| 53C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 80-540 | 99% | KT379958.1 | 0.0 |
| 55C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 90-630 | 99% | KU570318.1 | 2E-175 |
| 57C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 80-640 | 97% | KU570318.1 | 0.0 |
| 58C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas media* | 80-530 | 98% | KP813853.1 | 0.0 |
| 59C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 20-400 | 89% | KU942491.1 | 1E-197 |
| 66C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 20-540 | 94% | KR094132.1 | 0.0 |
| 74C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 20-430 | 96% | KT429783.1 | 0.0 |
| 86C | No Identification | *Aeromonas hydrophila* | 80-850 | 98% | KR094132.1 | 0.0 |
| 80C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas hydrophila* | 20-530 | 88% | KJ806448.1 | 0.0 |
| 92C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas media* | 80-640 | 99% | KF631429.1 | 0.0 |
| 95C | *Aeromonas hydrophila/caviae/ sobria 1* | *Aeromonas hydrophila* | 80-530 | 88% | HQ292702.1 | 1E-179 |
| 98C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas caviae* | 20-470 | 92% | KF835807.1 | 0.0 |
| 99C | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Aeromonas media* | 20-430 | 98% | KP967511.1 | 0.0 |
| 100C | Aeromonas hydrophila/caviae/ sobria 1/2 | *Aeromonas hydrophila* | 20-430 | 99% | KT361193.1 | 0.0 |

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4.4.1. Prevalence of confirmed *Aeromonas* spp. from bulk water and biofilm isolates by 16S rRNA sequencing.

*Aeromonas hydrophila* (73%) was the most prevalent species identified, followed *Aeromonas media* (20%) and *Aeromonas caviae* (7%). The results obtained in the present study are similar with data reported by other researchers in drinking water, where *A. hydrophila* is the predominant species from environmental isolates (Ghenghesh et al., 2001; Koksal et al., 2007; Razollini et al., 2008; Nashnoush et al., 2009; Al-sulami et al., 2014; Oleiwi et al., 2014).

**Table 4.4:** Percentage of *Aeromonas* spp. identities confirmed by 16S rRNA sequencing technique.

| ***Prevalence percentage of Aeromonas* isolates *confirmed by 16S rRNA sequencing*** | | | |
|---|---|---|---|
| **Bulk water isolates: n=12 (15%)** | | **Biofilm: n=18 (23%)** | |
| *River* | *Tap* | *River* | *Tap* |
| **8 (10%)** | **4 (5%)** | **14 (18%)** | **4 (5%)** |

The recovery of *Aeromonas hydrophila* as a predominant strain amongst other *Aeromonas* spp., from drinking water in this study presents a serious health concern. Given the fact that *A. hydrophila* is considered as the most commonly strain associated with a variety of infections in humans, including gastroenteritis, wound infections, septicaemia, haemolytic uremic syndrome, respiratory tract infections, meningitis, peritonitis and ocular infections (Galindo et al., 2006). *Aeromonas hydrophila* has also been suggested as one of the contaminants of concern in waterborne diseases by the World Health Organisation (WHO, 2004). It has been shown that *Aeromonas hydrophila* secretes many extracellular virulence factors which are involved in pathogenesis, such as haemolytic toxins, proteases and enterotoxin (Tomas, 2012). Therefore, isolation of *A. hydrophila* from household drinking water present a serious health threat to the rural communities who depend on such water for drinking and other household purposes. The water users might easily get exposed to the *A. hydrophila* strains through ingestion of such contaminated water for their daily survival and healthy living.

The recovery of other strains within the genus *Aeromonas* in the present study including *A. caviae*, and *A. media* from households' drinking water also signifies a health risk to the water users, since these species have been previously associated with human gastroenteritis



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(Pablos *et al.*, 2009). According to the WHO guidelines for drinking water, the presence of *Aeromonas* bacterial species in drinking water could be either due to insufficient treatment, post treatment contamination or the presence of excessive nutrients (WHO, 2011). In addition, other studies stated that the presence of *Aeromonas* spp. in water distribution systems suggests inadequate chlorine residuals or the potential for biofilms that lead to regrowth within pipes (Fernandez et al., 2000; September, 2007). Consequently, the occurrence of potential pathogenic *Aeromonas* spp. in this present study might be due to the ineffective treatment of drinking water at source point, which may have enhanced the survival of such bacteria as suspended or as part of the biofilm community within the storage containers.

The data in the present study clearly indicate high occurrence of *Aeromonas* species from drinking water obtained from river households' storage containers than tap households' storage containers. This might be due to the fact that raw water or water from the river source does not have residual disinfectant and therefore it is not able to limit any possible development of after-growth. The occurrence of *Aeromonas* spp. in this study thus emphasizes *Aeromonas* as one of the bacterial species that can be transmitted through drinking water. The data also further support the findings that suggest that the microbiological quality of treated water can deteriorate due to contamination during distribution or inadequate hygienic conditions in storage containers (September et al., 2007). *Aeromonas* species have been detected in treated water in several studies due to regrowth influenced by various factors (Koksal et al., 2007; September et al., 2007; Lautenschlager et al., 2010). Such factors includes low disinfection chlorine level, availability of carbon, nitrogen and phosphorus as nutrient source; since these factors are known to favour the biofilm formation in drinking water distribution systems which in turn provide favourable growth conditions for *Aeromonas* species. Literature also stated that biofilms play a role in protecting bacteria from residual chlorine in water distribution systems or treated drinking water, even possibly potential pathogenic bacteria (Wingender and Flemming, 2011).

In general, the recovery of *Aeromonas* bacterial species from drinking water in this current study can be considered as a health risk concern, as ingestion of such water may lead to associated bacterial infections. According to USEPA, (2002), immunocompromised individuals mostly vulnerable to such potential pathogenic bacteria include infants, young children, pregnant women, the very elderly, and those who have a severely weakened immune system or other major impairment of the host defence. Data from a study done in rural communities in South Africa revealed frequent association of *Aeromonas* spp. in cases of



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diarrhoea in HIV patients (Obi et al., 2007). Therefore, the high prevalence of *Aeromonas* spp. in this study present a significant health risk to the immunocompromised individuals in rural communities.

### 4.4.2 Opportunistic bacterial pathogens identified using 16S rRNA sequencing from presumptive positive bulk water and biofilm isolates

The following bacterial species were also identified using 16S rRNA gene sequences. These included *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Alcaligenes* spp., *Raoultella* spp., and *Morganella* species. Similar to the findings in the present study, several other studies revealed the presence of such bacterial organisms from drinking water sources. *Pseudomonas* spp., *Acinetobacter* spp., as well as *Aeromonas* bacterial spp. has been frequently identified in drinking water distribution systems (Norton and LeChevallier, 2000; Ribas et al., 2000; Mulamatatthil et al., 2014a). In addition, *Enterobacter cloacae* and *Klebsiella* species were amongst the most predominant bacterial species frequently isolated from drinking water distribution systems. Other species such as *Alcaligenes* spp. were also observed from distribution water, raw water, drinking water and distribution water during a chlorine failure (LeChevallier et al., 1980).

*Enterobacter* spp., *Klebsiella* spp., *Morganella morganii* and *Pseudomonas* spp. are well known members of the family *Enterobacteriaceae*. Most species of the family *Enterobacteriaceae* are known opportunistic bacteria responsible for several diseases such as diarrhoea, extra-intestinal infections, bacteraemia, and respiratory and urinary infections under certain conditions (Pollack, 2000; Lee and Liu, 2006; Abbott, 2007). The detection of these *Enterobacteriaceae* bacterial species in the present study may have interfered in the detection of *Aeromonas* isolates from both water and biofilm samples. These may be due to the limitation of required or selective nutrients for *Aeromonas* spp. growth, or harsh environmental conditions, and the presence of competing or fast-growing bacteria (Figueras et al., 2000).

*Pseudomonas aeruginosa* is a well-known opportunistic pathogen in humans. It has been predominantly found in aquatic environments especially in fresh surface water. This species been recognised as the enteric pathogen due to its ability to produce enterotoxins responsible for diarrhoea in children and infants. It has been also involved in nosocomial infections (WHO, 2003). *Pseudomonas aeruginosa* may cause a wide range of infections particularly in immunocompromised individuals such as urinary tract infections, respiratory diseases, ear



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and eye infections as well as bacteraemia, osteomyelitis and meningitis systemic diseases (Pollack, 2000). *Aeromonas* spp. and *Pseudomonas aeruginosa* are considered as the typical biofilm organisms due to the fact that they can pass water treatment barriers in very low densities and grow within water distribution systems where they lead to biofilm formation (WHO, 2003). Thus, the detection of *Pseudomonas aeruginosa* in particular is of health concern as literature has indicated its ability to form biofilms is characterised with greater protection against the host immune defense systems as well as its ability to provide susceptibility to various antibiotics (Leid et al., 2009).

*Enterobacter* spp. are increasingly encountered in nosocomial infections such as urinary tract infections and bacteraemia (Grimont and Grimont, 2006). Species of *Enterobacter* are also occasionally involved in community-acquired infections due to the widespread use of antibiotics responsible for community-acquired infections (Grimont and Grimont, 2006). *Enterobacter* species were found to be the second most common Gram-negative organism involved in nosocomial bloodstream infections in the Surveillance and Control of Pathogens of Epidemiological Importance project done in USA from 1995–2002 (Lockhart et al., 2007). According to the National Healthcare Safety Network report in USA, *Enterobacter* species were found to be the eighth most common cause of healthcare-associated infections based on the results obtained from a study on healthcare-associated infections in the period between 2006 and 2007 (Hidron et al., 2008).

*Klebsiella* spp. refers to common pathogens for community-acquired pneumonias, urinary tract infection, wound infections and bacteremias, with approximately 56% of *Klebsiella* infections being hospital-aquired (Janda and Abbott, 2006). *Klebsiella pneumoniae* has been identified as the most pathogenic strains to humans amongst all *Klebsiella spp.* (Podschun and Ullmann, 1998), and it has also been recognized as an important opportunistic pathogen to immunocompromised individuals (Janda and Abbott, 2006). Members of *Klebsiella* spp. are non-motile straight rods, oxidase-negative, catalase-positive *Enterobacteriaceae*, which are also commonly found in the intestinal tract of human. *Klebsiella* spp. is the causative agents of human infections such as asymptomatic colonization of the intestinal, urinary, or respiratory tract that can result in fatal septicaemia (Grimont et al., 2003; Brisse et al., 2006). *Klebsiella* spp. have been found in a variety of environmental samples, such as soil, vegetation, and water (Janda and Abbott, 2006).

*Acinetobacter* spp. is presently recognized to play a crucial role in a variety of nosocomial infections which includes bacteraemia, urinary tract infections and secondary meningitis. Members of the genus *Acinetobacter* are non-motile Gram-negative, strictly aerobic, non-



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fermentative, oxidase negative and catalase positive bacteria. In addition, *Acinetobacter* species are one of the leading causes of nosocomial pneumonia. However, only few of the commonly used antibiotics are reliable effective against such infections because *Acinetobacter* spp. is increasingly resistant to various antibiotics (Bergogne and Towner, 1996; Visca et al., 2011).

*Morganella morganii* is one of the recognized commensal organisms also known to cause urinary tract infections, respiratory system and blood infections in humans, though they have only been infrequently recovered from such source (Manos and Belas, 2006). *Morganella morganii* is motile, Gram-negative bacteria with peritrichous flagella (Lee and Liu, 2006; Manos and Belas, 2006). Bacteraemia due to *Morganella morganii* occurs once in a while. Furthermore, *Morganella morganii* has been reported to play a pathogenic role in pneumonia, empyema, pyomyositis, endophthalmitis, surgical wound infection, neonatal sepsis, and spontaneous bacterial peritonitis (Lee and Liu, 2006).

*Alcaligenes faecalis* is an opportunistic potential causes of infectious diseases often isolated from clinical cases (Chandhuri, 1967; Bizet and Bizet, 1997; Ashwath and Katner, 2005). *Alcaligenes spp.* is strictly aerobic, motile, Gram-negative, oxidase positive, non-fermentative bacteria that are widespread in aquatic environments (Bizet and Bizet, 1997; Anderson et al., 2003).

*Raoultella ornithinolytica* has been associated with few cases of bacteraemia and thus indicate its pathogenic role as a blood borne human pathogen (Mau and Ross, 2010; Hadano et al., 2012; Haruri et al., 2014). Members of the genus *Raoultella* are nonmotile, Gram-negative oxidase-negative (Chun et al., 2015) and naturally widespread in aquatic environments (Drancourt et al., 2001).

The high prevalence of *Enterobacter* spp. in this study is in agreement with a study done by Obi et al., (2005) in various water sources in the Venda Region of the Limpopo Province, where *Enterobacter cloacae, Aeromonas hydrophila, Aeromonas caviae* were detected in water with a higher prevalence in the months of summer in comparison to other seasons ($p<0.05$). Opportunistic bacterial spp. identified in the present study was also observed in several studies done in other countries in drinking water. Shittu et al., (2008) showed the presence of *Enterobacter aerogenes, Pseudomonas* spp., *and Klebsiella* spp. from drinking water of Abeokuta, Nigeria. Mwajuma, (2010) recovered *Aeromonas hydrophila*, *Klebsiella* and *Pseudomonas* spp. from selected drinking water sources in Samburu South. Omezuruike et al., (2008) also identified *Pseudomonas aeruginosa, Enterobacter aerogenes, Klebsiella*



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species, and *Acinetobacter* species from different drinking water samples of Abeokuta and Ojota, Lagos state Nigeria. *Pseudomonas aeruginosa* was also detected in different drinking water resources of Makkah City, Saudi Arabia (Saati and Faidah, 2013).

Opportunistic bacterial species identified in this study, such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Enterobacter spp. and Acinetobacter spp.* including *Aeromonas* spp. have been previously detected within biofilms in drinking water distribution systems due to regrowth in water distribution systems (USEPA, 2002). *Aeromonas* and *Pseudomonas aeruginosa* has been associated with biofilms and as a result minimise the effect of disinfectants. The occurrence of *Pseudomonas, Aeromonas, Klebsiella, Enterobacter, Acinetobacter,* and *Alcaligenes* in drinking water has been previously reported (Schindler and Metz, 1991; Momba et al., 2000). Therefore, the occurrence of these opportunistic bacterial pathogens in this study presents a health risk particularly to the immunocompromised individuals in rural communities.



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**Table 4.5:** Opportunistic bacterial species identified by 16S rRNA sequencing not in accordance with the API 20E identification technique

| Isolates | API 20E ID | Sequencing ID | Nucleotides used | Similarity | Accession number | E value |
|---|---|---|---|---|---|---|
| **Bulk Water:** | | | | | | |
| **2B** | *Aeromonas hydrophila/caviae/ sobria 1* | *Enterobacter cancerogenus* | 20-640 | 98% | KJ410179.1 | 0.0 |
| **10B** | *No Identification* | *Enterobacter cloacae* | 80-540 | 98% | HQ179578.1 | 0.0 |
| **16B** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter ludwigii* | 20-630 | 75% | KJ42458.1 | 1E-109 |
| **19B** | *No Identification* | *Enterobacter asburiae* | 80-740 | 95% | KR025379.1 | 0.0 |
| **25B** | *No Identification* | *Enterobacter asburiae* | 70-580 | 84% | KF285976.1 | 2E-73 |
| **29B** | *No Identification* | *Enterobacter cancerogenus* | 80-540 | 97% | KJ410179.1 | 0.0 |
| **30B** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cancerogenus* | 10-590 | 75% | KM279661.1 | 3E-126 |
| **31B** | *No Identification* | *Morganella morganii* | 20-750 | 76% | KJ626252.1 | 1E-151 |
| **38B** | *No Identification* | *Klebsiella pneumoniae* | 80-640 | 93% | KU254764.1 | 0.0 |
| **40A** | *Aeromonas hydrophila/caviae/ sobria 2* | *Klebsiella pneumoniae* | 80-640 | 96% | KT630867.1 | 0.0 |
| **41B** | *No Identification* | *Klebsiella pneumoniae* | 90-640 | 98% | KU711922.1 | 0.0 |
| **43B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Enterobacter hormaechei* | 20-530 | 96% | KP236341.1 | 0.0 |
| **42B** | *Aeromonas hydrophila/caviae/ sobria 1* | *Enterobacter cloacae* | 80-660 | 98% | KU051718.1 | 0.0 |
| **46B** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Klebsiella pneumoniae* | 80-550 | 80% | KU321221.1 | 1E-99 |
| **50B** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Raoultella ornithinolytica* | 80-640 | 95% | KT378441.1 | 0.0 |
| **52B** | *Aeromonas hydrophila/caviae/ sobria 1* | *Enterobacter cancerogenus* | 20-530 | 83% | KJ410179.1 | 0.0 |
| **53A** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cloacae* | 20-470 | 97% | KT873250.1 | 0.0 |
| **54B** | *Aeromonas hydrophila/caviae/ sobria 1* | *Enterobacter hormaechei* | 30-440 | 95% | KT380664.1 | 0.0 |
| **58B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Enterobacter cancerogenus* | 100-530 | 97% | KJ410179.1 | 0.0 |
| **59B** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cloacae* | 100-640 | 98% | KT441077.1 | 0.0 |
| **62B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Enterobacter cloacae* | 40-640 | 97% | KU936831.1 | 0.0 |
| **64B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Acinetobacter soli* | 80-320 | 86% | KT387351.1 | 1E-72 |
| **69B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Enterobacter cloacae* | 20-530 | 85% | KP226582.1 | 0.0 |
| **78B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Enterobacter spp* | 20-740 | 82% | KC835101.1 | 0.0 |

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| | | | | | | |
|---|---|---|---|---|---|---|
| **79B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Klebsiella pneumoniae* | 20-540 | 91% | KP844457.1 | 0.0 |
| **85B** | *Aeromonas hydrophila/caviae/ sobria 2* | *Klebsiella pneumoniae* | 30-530 | 87% | KT943576.1 | 0.0 |
| **89B** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Alcaligenes faecalis* | 80-460 | 91% | KM251283.1 | 2E-150 |
| **Biofilm:** | | | | | | |
| **8C** | *No Identification* | *Klebsiella pneumoniae* | 80-640 | 99% | KU711922.1 | 0.0 |
| **9C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Pseudomonas aeruginosa* | 80-630 | 89% | KC505184.1 | 0.0 |
| **10C** | *No Identification* | *Enterobacter cloacae* | 80-640 | 99% | KU570360.1 | 0.0 |
| **16C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cloacae* | 80-540 | 96% | KJ607177.1 | 0.0 |
| **29C** | *Aeromonas hydrophila/caviae/ sobria 2* | *Enterobacter asburiae* | 20-530 | 99% | HQ242719.1 | 0.0 |
| **30C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cancerogenus* | 80-530 | 94% | KJ410179.1 | 0.0 |
| **37C** | *Aeromonas hydrophila/caviae/ sobria 1* | *Enterobacter asburiae* | 20-740 | 83% | KM019904.1 | 0.0 |
| **40C** | *No Identification* | *Klebsiella pneumoniae* | 80-510 | 97% | KU674964.1 | 0.0 |
| **42C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cloacae* | 90-640 | 99% | KU936831.1 | 0.0 |
| **43C** | *Aeromonas hydrophila/caviae/ sobria 1* | *Pseudomonas aeruginosa* | 20-550 | 81% | HQ122657.1 | 1E-83 |
| **45C** | *No Identification* | *Enterobacter hormaechei* | 80-540 | 98% | KP729442.1 | 0.0 |
| **49C** | *Aeromonas hydrophila/caviae/ sobria 1* | *Enterobacter cloacae* | 80-550 | 96% | KF932264.1 | 0.0 |
| **51C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Acinetobacter seifertii* | 80-640 | 99% | NR134684.1 | 0.0 |
| **52C** | *Aeromonas hydrophila/caviae/ sobria 1* | *Pseudomonas aeruginosa* | 100-500 | 100% | KT45648.1 | 0.0 |
| **60C** | *No Identification* | *Pseudomonas pseudoalcaligenes* | 80-430 | 83% | JN717222.1 | 0.0 |
| **61C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter kobei* | 20-560 | 72% | KM593927.1 | 2E-89 |
| **79C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cancerogenus* | 80-530 | 84% | KT261416.1 | 0.0 |
| **70C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter spp* | 20-480 | 75% | HM461157.1 | 0.0 |
| **82C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter hormaechei* | 10-190 | 83% | KP729442.1 | 1E-31 |
| **90C** | *No Identification* | *Enterobacter cloacae* | 80-530 | 100% | KT441077.1 | 0.0 |
| **96C** | *Aeromonas hydrophila/caviae/ sobria 1/2* | *Enterobacter cloacae* | 80-750 | 99% | KU936831.1 | 0.0 |

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The data clearly show that not all isolates identified as presumptive positive *Aeromonas* spp. by biochemical identification method were confirmed as *Aeromonas* species using molecular identification system as shown in Table 4.5. However several other opportunistic bacterial species were identified using 16S rRNA sequencing. Biochemical tests have indicated that these other bacterial species were potentially also *Aeromonas* species. This could be due to the lack of uniformity and atypical reactions of some biochemical characteristics that may have led to misidentification of bacterial species. Amongst all bacterial species identified in this study using 16S rRNA molecular method, *Enterobacter* species were found to be the most prevalent species identified from *Aeromonas* presumptive positive isolates with (31; 40%), followed by *Aeromonas* species (30; 38%), *Klebsiella* species (8; 10%), *Pseudomonas* species (4; 5%), *Acinetobacter* species (2; 3%) as well as 1 (1%) each of *Alcaligenes faecalis*, *Morganella morganii* and *Raoultella ornithinolytica* respectively (Figure 4.5).

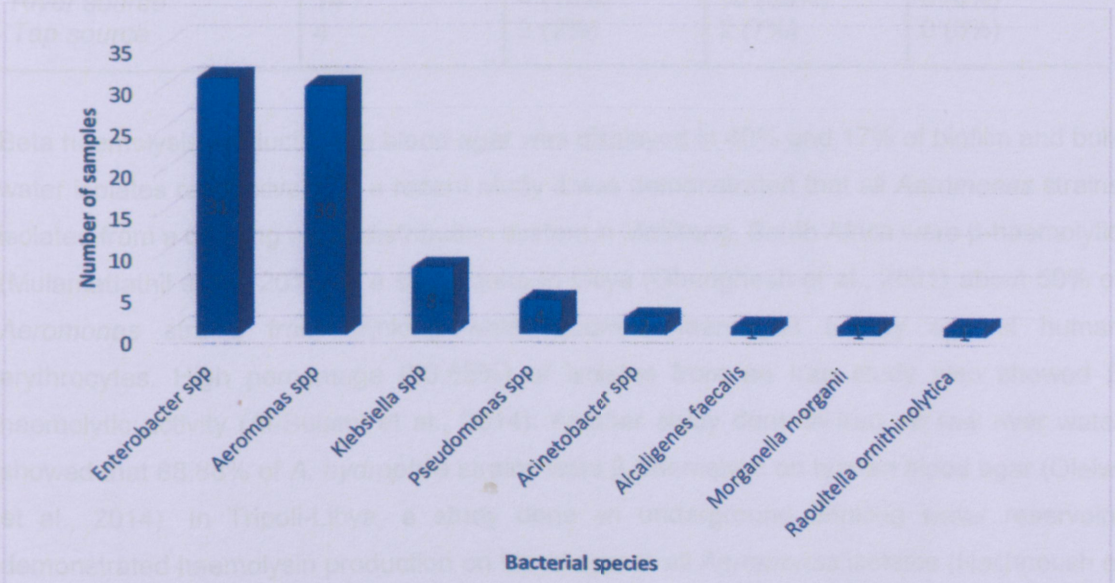



**Figure 4.3:** A column chart indicating the prevalent of all bacterial species identified from both bulk water and biofilm isolates using 16S rRNA sequencing identification technique.

### 4.5. Haemolysin production

The haemolysin production test was done to determine the pathogenic potential of the confirmed *Aeromonas* isolates. Only Gram negative, oxidase positive and API 20E *Aeromonas* identified samples that were also confirmed by 16S rRNA sequencing as *Aeromonas* species (which included 12 bulk water and 18 biofilm isolates) were tested further for haemolysis production. None of the tested isolates displayed γ-haemolysis. A total of 43%



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isolates (bulk water and biofilm) demonstrated α-haemolysis which was indicated by the greenish discolouration or darkening of the blood agar surrounding the streaked bacterial colonies. A total of 57% of both bulk water and biofilm isolates demonstrated β-haemolysis which was indicated by a clear zone around the bacterial colony on blood agar. Haemolysin production results are shown in Table 4.6.

**Table 4.6:** Haemolysin production activity for bulk water and biofilm isolates tested

| Isolates | Number tested (n=30) | α haemolysin n=13 (43%) | β haemolysin n=17 (57%) | γ haemolysin n=0 (0%) |
|---|---|---|---|---|
| Bulk water (total) | 12 | 7 (23%) | 5 (17%) | 0 (0%) |
| *River source* | 8 | 3 (10%) | 5 (17%) | 0 (0%) |
| *Tap source* | 4 | 4 (13%) | 0 (0%) | 0 (0%) |
| Biofilm (total) | 18 | 6 (20%) | 12 (40%) | 0 (0%) |
| *River source* | 14 | 4 (13%) | 10 (33%) | 0 (0%) |
| *Tap source* | 4 | 2 (7%) | 2 (7%) | 0 (0%) |

Beta haemolysis production on blood agar was displayed in 40% and 17% of biofilm and bulk water isolates respectively. In a recent study it was demonstrated that all *Aeromonas* strains isolated from a drinking water distribution system in Mafikeng, South Africa were β-haemolytic (Mulamattathil et al., 2014).In a study done in Libya (Ghenghesh et al., 2001) about 50% of *Aeromonas* strains from drinking water showed haemolytic activity against human erythrocytes. High percentage (83.65%) of isolates from an Irag study also showed β haemolytic activity (Al-Sulami et al., 2014). Another study done in Iraq on raw river water showed that 88.88% of *A. hydrophila* strains were β-haemolytic on human blood agar (Oleiwi et al., 2014). In Tripoli-Libya, a study done on underground drinking water reservoirs demonstrated haemolysin production on blood agar in all *Aeromonas* isolates (Nashnoush et al., 2009). In India, 71% of environmental isolates from water sources that were regularly used for domestic purposes produced haemolysin (Bhowmilk et al., 2009). Eighty nine percent (89%) of domestic water samples in Chenai also showed β-haemolytic activity (Alavandi and Ananthan, 2003). Moreover, majority of the isolates displayed haemolytic activity against sheep erythrocytes in a study done on tap, mineral and well drinking water (Scoaris et al., 2008).

Beta haemolytic activity observed in *Aeromonas* isolates tested in the present study is lower in comparability with several studies conducted on environmental samples. Such difference from the present study data for well-known β-haemolytic *Aeromonas* species might be due to



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the type of blood agar used. Another explanation can be also due to the lower incubation period to clear all the red blood cells in the blood agar.

The recovery of both bulk water and biofilm isolates with the ability to display β-haemolysin toxins present a health risk to the rural community depending on such water for human consumption. Since β-haemolysin is a virulence factor that belongs to a group of pore-forming bacterial cytolysins and that usually interferes with the normal integrity of the host cell membrane leadings to the leakage of the cytoplasmic contents (Abrami et al., 1998). In addition, the production of the β-haemolysin toxin in motile *Aeromonas* isolates has been reported as one of the virulence factor of *Aeromonas* species that is well known to be associated with gastrointestinal disorder (Majeed and MacRae, 1993, Erova et al., 2007).

Alpha haemolysis on blood agar was displayed in 20% and 23% of biofilm and bulk water isolates respectively. No known local documented data on α haemolysin production of *Aeromonas* strains isolates from drinking water could be found. Although earlier studies have shown that enterotoxin is produced by most β-haemolytic strains of *Aeromonas,* the data from a study done in India on clinical and environmental isolates revealed that the capacity for enterotoxin production in *Aeromonas* spp. is not only confined to the β-haemolytic strains (Singh and Sanyal, 1992a). The study reported that α and non-haemolytic isolates also possess haemolytic activity properties, although to a lesser extent regardless of their species designation and source of isolation. It may therefore cause a secretory response in the gut (Singh and Sanyal, 1992b).

Moreover, it is possible that the non-haemolytic isolates on blood agar may possess the haemolytic gene either that could not be expressed or that had mutations affecting domains responsible for the haemolytic phenotype (Wang et al., 2003). This means that about 50% of the isolates which produced α-haemolysin toxins in this study should not be undermined, as they could also play a role in pathogenesis.

**4.6. Molecular detection of virulence genes from bulk water and biofilm isolates**

Both bulk water and biofilm isolates were screened for the presence of virulence genes to determine the pathogenic potential of isolates. Results are recorded in Table 4.7. The detection of virulence genes which express extracellular enzymes such as gelatinase and hyaluronidase and genes that express haemolytic activity inclusive of cytolysin and asa1 were used as the direct way to reveal the potential pathogenicity of the isolates. A total of 30 isolates (18 biofilm and 12 bulk water) which were confirmed as *Aeromonas* strains by 16S rRNA



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sequencing were used for the determination of virulence genes. The cylA and *hyl* genes could not be detected in any of the isolates. However, 47% of biofilm isolates tested positive for the *GelE* gene (Figure 4.6 A), while 33% were positive for the *asa1* virulence gene (Figure 4.6 B). On the other hand, among the bulk water isolates 20% tested positive for the *GelE* gene and 17% for the *asa1* gene (Table 4.7).

**Table 4.7:** Number of confirmed *Aeromonas* isolates indicating amplified targeted virulence factors genes using singleplex PCR.

| Virulence genes tested | Virulence factor | Bulk Water | Biofilm | Overall of Bulk Water and Biofilm (n=30) |
|---|---|---|---|---|
| *Asa1* | Aggregation substance | 5 (17%) | 10 (33%) | 15 (50%) |
| *cylA* | Cytolysin | 0 (0%) | 0 (0%) | 0 (0%) |
| *gelE* | Gelatinase | 6 (20%) | 14 (47%) | 20 (67%) |
| *hyl* | Hyaluronidase | 0 (0%) | 0 (0%) | 0 (0%) |

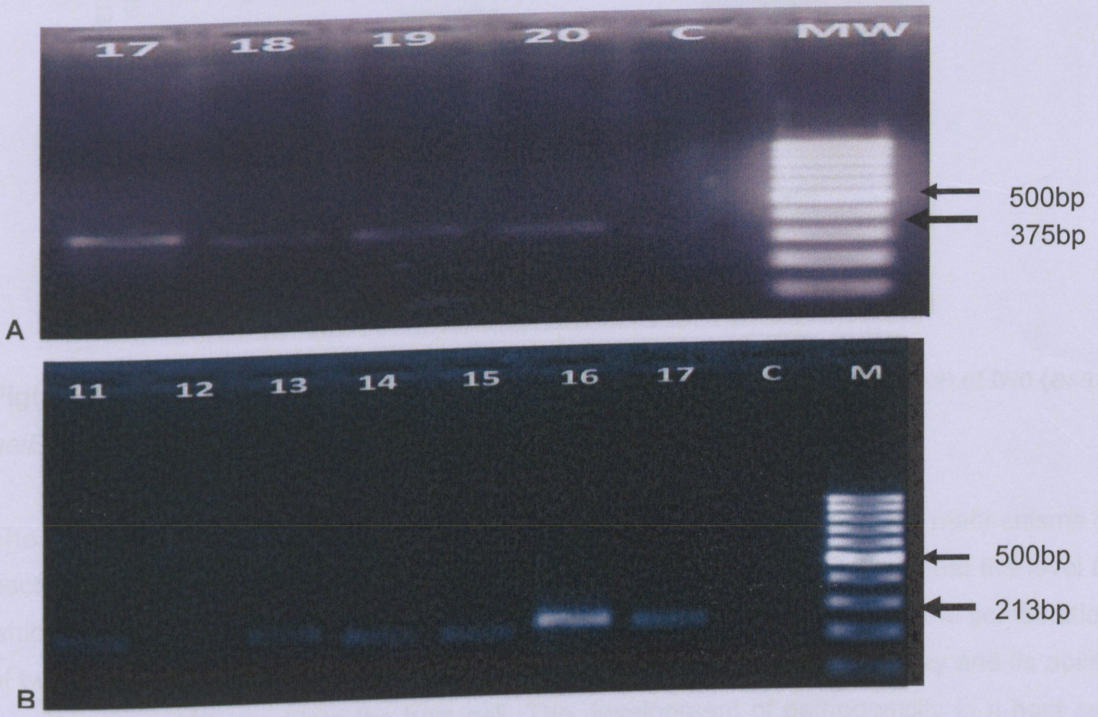



**Figure 4.4:** 1% (w/v) agarose gels stained with gel red representing water and biofilm isolates indicating the amplified product of (A) *Asa1* gene at 375bp and (B) *gelE* gene at 213bp.



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The *gelE* virulence gene is associated with the production of the extracellular enzyme gelatinase involved in *Aeromonas* pathogenicity was observed in 67% in all isolates. The *asa1* gene associated with haemolytic activity of *Aeromonas* strains was observed in 50% of all isolates (bulk water and biofilm). A total of 4 biofilm isolates and only 1 bulk water isolate in this study were found with at least one virulence gene (*gelE*) factor as illustrated in Figure 4.7. A combination of *gelE* virulence gene and *asa1* virulence gene was observed in 4 bulk water and 10 biofilm isolates. However, literature has indicated that potential pathogenic bacteria usually make use of the presence of two main virulence factors for the development of pathogenicity in the host cell (Madigan et al., 2009). Therefore, detection of more than one virulence factor indicates the pathogenic potential of *Aeromonas* strains isolated from household drinking water in this study.




**Figure 4.5:** Illustration of distribution of 1 virulence gene (*gelE*) and combination of two (*asa1*; *gelE*) virulence genes amongst bulk water and biofilm isolates

The detection of virulence factors is essential for acquiring information on the mechanisms of bacterial pathogenesis (Wu et al., 2011; Casadevall et al., 2011). However, that the level to which a virulence factor causes harm to a host cell does not only depend on the combination of two or more virulence genes in one bacterial species, but also on its toxicity and its ability to penetrate and spread into the host cell. The development of pathogenicity in a host cell further depends on the host cell immune status (Madigan et al., 2009).

Gelatinase is responsible for hydrolysis of gelatin, which leads to the loss of gels attributes and conversion to liquid form even at low temperatures and thus indicate its proteolytic activity



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(Thornley et al., 1997). Gelatinase has been recognised to cause host tissue damage, allows colonization and also promote establishment of infection by overcoming the host defense and thus has been recommended for evaluation of protease activity in *Aeromonas* species (Castro-Escarpulli et al., (2003). In the present study, *gelE* gene was observed in 20% bulk water and 47% biofilm, with an overall prevalence of 67%. Gelatinase activity was also reported among 34% of *Aeromonas* isolates obtained from environmental water samples in South India (Indra et al., 2016). In another study conducted in Canada gelatinase activity was reported in 96.9% on strains isolated from different origins including environmental, food and clinical (Albarral et al., 2015).

The detection of gelatinase activity in drinking water isolates in the present study present a health threat. A study done on environmental isolates showed that gelatinase activity was significantly correlated with cytotoxin production. The exact role of gelatinase in *in vivo* scenarios are not completely understood (Bhowmilk et al., 2009). Cytotoxin production by *Aeromonas* species might results in gastrointestinal infections in humans due to the enterotoxin properties they possess. Moreover, enterotoxin activity of pathogenic *Aeromonas* species allows degeneration of crypts and villi of the small intestine which usually lead to human intestinal disorders (Tomas, 2012; Pandove et al., 2013).

Hyaluronidase (*hyl*) is an enzyme that is believed to be used as a virulence factor by some pathogenic bacteria to destroy or degrade the polysaccharides that holds the host cells together. Thus, this makes it easier for the pathogenic bacteria to spread through the tissues of the host organism (Todar, 2002). However, there is no valid experimental data available to support this hypothesis. In the present study none of the isolates tested positive for the *hyl* gene (Table 4.7).

Aggregation substance refers to a virulence factor encoded by *asa1* gene. It is considered as one of the haemolysin enzymes that play a fundamental role in *Aeromonas hydrophila* pathogenesis (USEPA, 2006; Rasmussen-Ivey et al., 2016). In the present study, *asa1* gene was observed in 17% bulk water isolates and 33% biofilm isolates, with an overall prevalence of 50%. Higher detection rates (76.78%) had been reported in *A. sobria* isolates (Das et al., 2013). Wang et al., (2003) observed that 67% of *A. veronii biovar sobria* isolates were positive for haemolysin *(asa1)* genes. Haemolysins production by *Aeromonas* species indicate their haemolytic activity which is one of the known important virulent factor of *Aeromonas* species. This enzyme is involved in the degradation of the host red blood cells (Yadav et al., 2014).



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Thus, detection of *asa1* gene in the present study might be associated with haemolytic activity of the isolates from the contaminated drinking water.

Cytolysin refers to virulence factor encoded by *cylA* that possess bactericidal properties and beta-haemolytic effects in human red blood cells, and its activity contributes to severity of infection. Cytolysins (*cylA*) are responsible for lysis or destruction of red blood cells by releasing haemoglobins. None of the isolates in this study tested positive for the cytolysin virulence gene. These findings are in agreement with those reported by Sechi et al., (2002), where no cytotoxic properties were detected in environmental strains. In contrast to the present study, several enterotoxins virulence genes that contribute to gastroenteritis in humans and animals have been detected in drinking water utilities (USEPA, 2006).

Cytolysin gene expression is usually concurrent with haemolysin production (Chopra, 2008). In contrast to the findings in the present study, a correlation between the haemolysin production on blood agar and cytolysin genes responsible for haemolytic activity in *Aeromonas* species was not observed (Chopra, 2008). However, in this study the cytolysin gene was not detected in any of the isolates (bulk water and biofilm). Beta haemolysis was observed in 57% of all bulk water isolates and α-haemolysin was observed in 43% of bulk water and biofilm isolates. This could be due to the fact that the *cylA* is not strongly conserved as one of the *Aeromonas* isolates genes responsible for expression of haemolytic activity. The isolates may use another gene possessing the role of haemolytic activity. Closely similarly to the present study and one conducted in India on environmental isolates observed that a majority of isolates (71%) had haemolytic activity. However, only few isolates were positive for the haemolysin gene using PCR assay (Bhowmilk et al., 2009).

Another explanation could be due to the failure of molecular experimental conditions used in this study which in turn affect for detection of gene known to express haemolysin activity. Thus, the virulence data in this study emphasizes the need for testing virulence factors phenotypically and genotypically. Moreover, it could be due to the environmental conditions of the isolates origin such as multiplication factor which alters the genes that express haemolytic activity (Yadav et al., 2014). The phenotypic and genctypic results obtained regarding haemolysin gene expression in the present study support findings that suggest that the haemolysin genes seems not to be distributed randomly among the different *Aeromonas* species (Wang et al., 2003).



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Not all of the targeted genes encoding for haemolytic activity were displayed in this study. However, data reported from several studies indicated that *Aeromonas* species from drinking water possess a variety of virulence factors genes associated with haemolytic and enterotoxin activity that usually lead to gastrointestinal and extra intestinal infections in humans (Sen and Rodgers, 2004; Scoaris et al., 2008; Robertson et al., 2014). Furthermore, findings of a wide variety of virulence related genes in a study done in US by Sen and Rodgers, (2004) suggests that municipal treated drinking water may be a source of potential pathogenic bacterial species (Sen and Rodgers, 2004). The data obtained in a study done in Turkey showed that 76% of *Aeromonas* isolates from river water exhibited beta haemolytic activity (Kivanc et al., 2011). Also, data from previous studies done on water samples have demonstrated the ability of *Aeromonas* species to produce exotoxins, which includes alpha and beta haemolysins, proteases, enterotoxin and acetylcholinesterase (Sen and Rodgers, 2004; Singh et al., 2009).

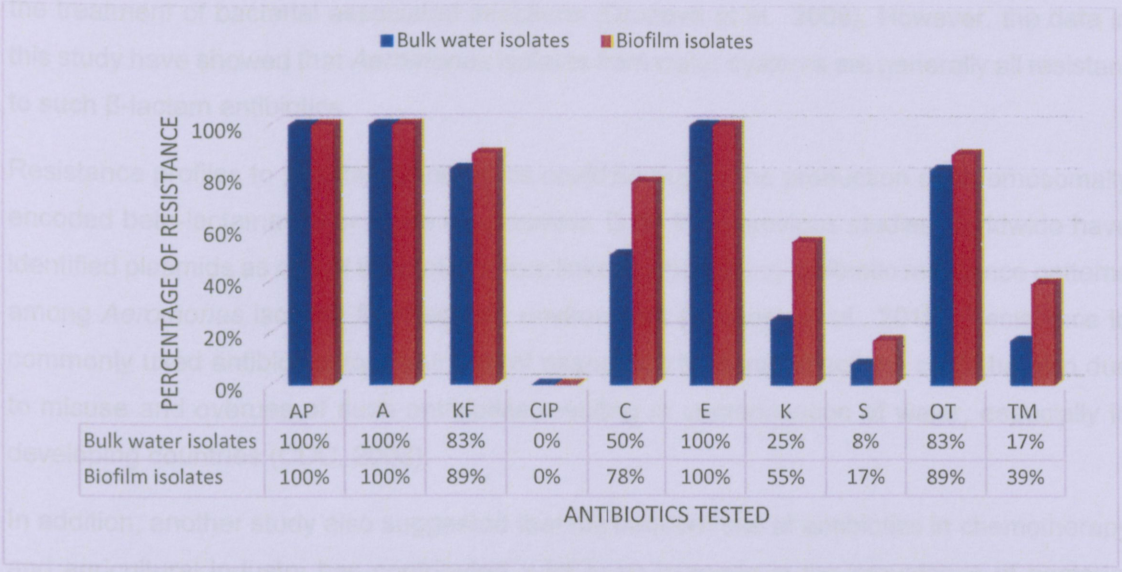
The presence of any virulence factor-encoding genes in *Aeromonas* water isolates in the present study should not be undermined. Such genes tested for could potentially be better expressed in human host cells and lead to devastating conditions in humans and animals (US USEPA, 2006). Therefore, the detection of gelatinase (*gelE*), aggregative substance (*asa1*) and production of haemolysins particularly β haemolysin from isolates in this present study indicate potential pathogenicity of *Aeromonas* strains isolated from household drinking water.

### 4.7. Antibiotic resistance patterns

The Kirby-bauer antibiotic resistance test was performed to all bulk water and biofilm isolates confirmed as members of the genus *Aeromonas* by 16S rRNA identification system. The antibiotic resistance test was done as described in Section 3.8 and isolates were tested against 10 antibiotics of clinical interest. Subsequently, phenotypic antibiotic resistance profiles for *Aeromonas* strains tested were generally determined according to the recommended Clinical and Laboratory Standards Institute (CLSI) guidelines for the *Enterobacteriaceae* (CLSI, 2011). Phenotypic antibiotic resistance profiles can be used for determination of effective treatment for identified *Aeromonas* strains associated infections



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| | AP | A | KF | CIP | C | E | K | S | OT | TM |
|---|---|---|---|---|---|---|---|---|---|---|
| Bulk water isolates | 100% | 100% | 83% | 0% | 50% | 100% | 25% | 8% | 83% | 17% |
| Biofilm isolates | 100% | 100% | 89% | 0% | 78% | 100% | 55% | 17% | 89% | 39% |

**Figure 4.6:** Antibiotic resistance patterns of bulk water and biofilm isolates from households' drinking water storage containers.

All isolates from both bulk water and biofilm in this study were resistant to ampicillin (AP) 10 µg (100%), amoxicillin (A) 10 µg of (100%), and erythromycin (E) 15 µg (100%) as indicated in Figure 4.8. This was followed by high numbers of isolates being resistant to cephalothin (KF 30 µg; 83-89%) and oxytetracycline (OT 30 µg; 83-89%). A large number of isolates were also resistant to chloramphenicol (C 10 µg; 50 and 78%). Resistance to kanamycin (K 30 µg) ranged between 25 and 55%. Resistance to trimethoprim (TM 2.5µg) ranged between 17 and 39%. Streptomycin 10µg resistance ranged between 8 and 17%. None or of the isolates were resistant to ciprofloxacin 5 µg. Overall, more biofilm isolates were resistant to the antibiotics tested compared to bulk water isolates.

Complete resistance of *Aeromonas* isolates to ampicillin (100%), amoxicillin (100%) and erythromycin (100%) observed in this study is in agreement with previous studies done in South Africa on drinking water that showed absolute resistance to these $\beta$-lactam and macrolides antibiotics (Mulamattathil et al., 2014; Obi et al., 2004). Similarly, Obi et al also observed a large number of isolates that were resistant to ampicillin, amoxicillin and erythromycin in *Aeromonas* species isolated from diarrhoeic stools of patients in Vhembe district, Limpopo, South Africa (Obi et al., 2007). In a study done in an aquaculture system in South Africa, it was also observed that a large number of isolates were resistant to erythromycin (Jacobs and Chenai, 2007). In addition, a study done on surface water by Ramalivhana, (2010) showed 98% of isolated were resistant to ampicillin. These observations present a health concern, since β-lactam antibiotics are the most widely used antibiotics for



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the treatment of bacterial associated infections (Orozova et al., 2008). However, the data in this study have showed that *Aeromonas* isolates from water systems are generally all resistant to such β-lactam antibiotics.

Resistance profiles to β-lactams antibiotics could be due to the production of chromosomally encoded beta-lactamases or those on plasmids. Data from previous studies worldwide have identified plasmids as one of the main factors linked to increasing antibiotic resistance patterns among *Aeromonas* isolates from aquatic environment (Igbinosa et al., 2012). Resistance to commonly used antibiotics for treatment of associated bacterial infections could be also due to misuse and overuse of such antibiotics resulting in contamination of water, especially in developing countries (CDC, 2003).

In addition, another study also suggested that the frequent use of antibiotics in chemotherapy and agricultural industry has contributed a lot in an increase in the prevalence of bacterial resistant strains to these antibiotics (Power, 2006). Furthermore, resistance could also be due to the previous exposure of isolates to antibiotic tested either from clinical wastewater or from any antimicrobial polluted water (Vila et al., 2003). Another explanation could be due to the production of several secreted enzymes including β-lactamases (metallo-β-lactamase, cephalosporinase, and penicillinase) by species of the genus *Aeromonas*, which inactivate β-lactam antibiotics and thus contribute to antibiotic resistance (Janda and Abbott, 2010).

Furthermore, all isolates in this study were susceptible to ciprofloxacin (100%), followed by most isolates susceptible to streptomycin. This is in agreement with the study done by Mulamattathil et al., (2014) in which only few isolates were resistant to streptomycin. This is also in consistent with a study conducted in isolates from river source in Kwazulu Natal, South Africa (Lin et al., 2004) and also in a study done in Mafikeng, South Africa on drinking water Mulamattathil et al., 2014). Therefore ciprofloxacin can be used as an antibiotic of choice for treatment of *Aeromonas* associated infections that can arise from any ingestion or contact of water. The antibiotic resistance pattern data observed in this study are therefore comparable to earlier resistance patterns documented in *Aeromonas* isolates in South Africa (Lin et al., 2004; Mulamattathil et al., 2014). Findings of *Aeromonas* strains resistance to a variety of antibiotics in South Africa is clearly of health concern considering the high incidence of HIV/AIDS cases documented (Dorrington et al., 2006).

The antibiotic resistance profiles observed in the present study are also consistent with other studies done on surface or raw water and drinking water distribution systems and reservoir in other countries. A study done in Iraq on drinking water also reported that all *Aeromonas* strains were resistant to ampicillin and all were susceptible to ciprofloxacin (Al-Sulami et al., 2014).



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In Portugal and in Lebanon all strains from untreated water used for drinking were resistant to amoxicillin (Carvalho et al., 2012; Tokajian and Haswa, 2004). In Egypt, all *Aeromonas* isolates from drinking water were resistant to ampicillin and sensitive to ciprofloxacin (Mahdi et al., 2008).

Similarly to the present study, data from a study done in Kenya on *Aeromonas* river isolates showed complete resistance to ampicillin (100%) with the most sensitive antibiotic shown to be ciprofloxacin (100%) (Njeru et al., 2012). In Libya, Tripoli, almost all isolates, except one isolate from drinking water were susceptible to ciprofloxacin and 75.6% isolates were resistant to ampicillin (Nashnoush et al., 2009). Ciprofloxacin was reported as the most effective antibiotic against *Aeromonas* isolates from drinking water and 92% of isolates were resistant to ampicillin (Scoaris et al., 2008).The data from previous studies support the findings in the present study that shows resistance of *Aeromonas* to a variety of antibiotics and thus the resistance data observed in this study is not uncommon.

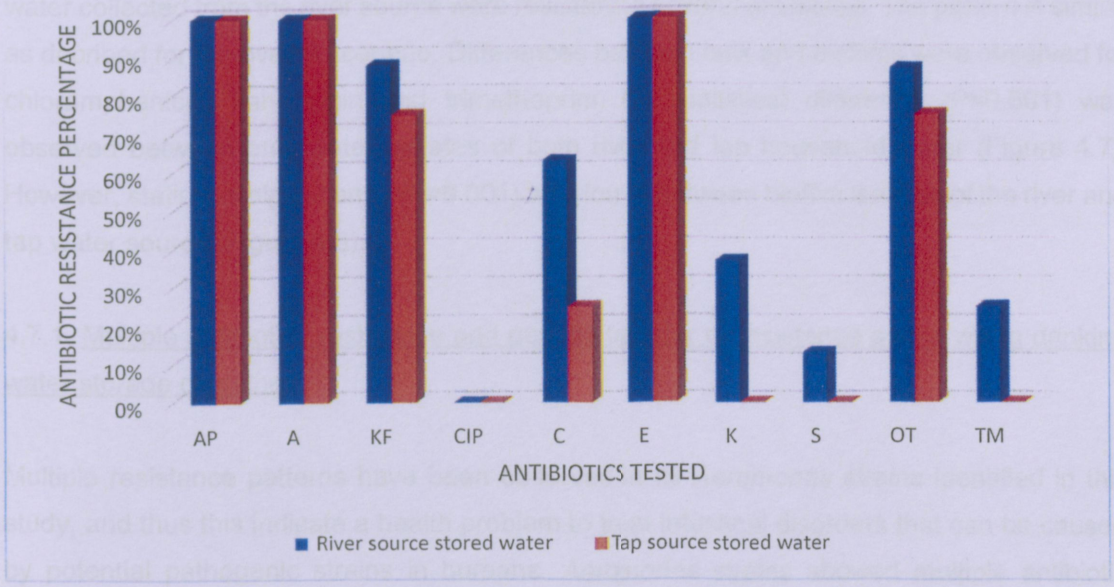



**Figure 4.7:** Comparison of antibiotic resistance patterns displayed from river and tap bulk water source isolates.



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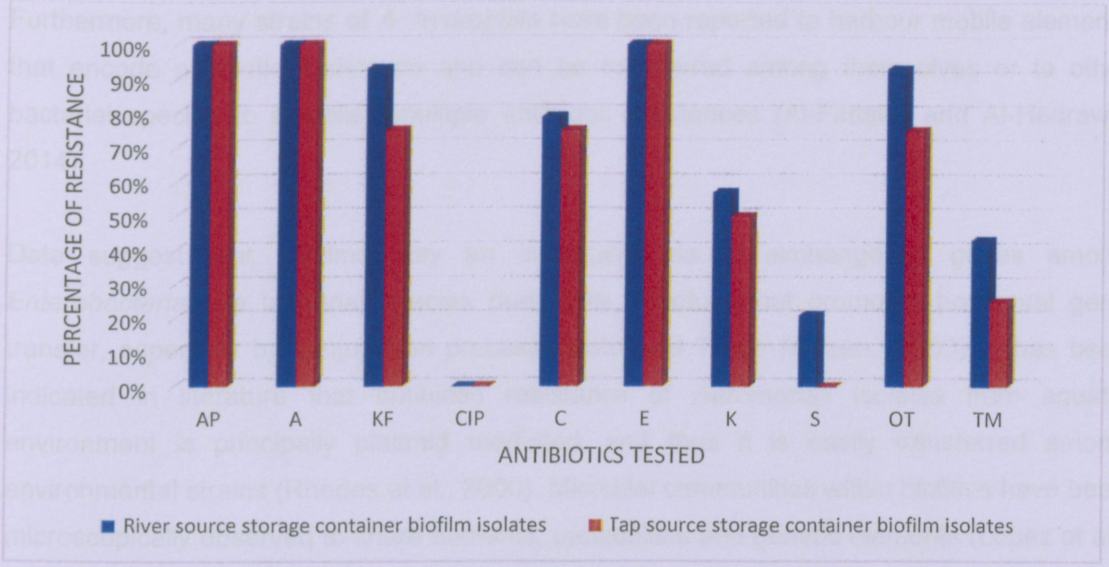



**Figure 4.8:** Comparison of antibiotic resistance patterns observed from biofilm isolates from storage containers with tap and river water.

Almost all bulk water isolates and biofilm isolates analysed from rural households' drinking water collected from the river source were resistant to several antibiotics. The pattern is similar as decribed for the overall scenario. Differences between bulk and biofilms were observed for chloramphenicol, kanamycin and trimethoprim. No statistical difference (P=0.601) was observed between bulk water isolates of both river and tap household water (Figure 4.7). However, statistical significance (P=0.001) was found between biofilm isolates of the river and tap water source (Figure 4.8).

### 4.7.1. Multiple antibiotics resistance and genetic transfer of resistance genes within drinking water storage containers

Multiple resistance patterns have been observed in all *Aeromonas* strains identified in this study, and thus this indicate a health problem to treat intestinal disorders that can be caused by potential pathogenic strains in humans. *Aeromonas* strains showed multiple antibiotic resistant patterns ranging from four to nine antibiotics. According to literature, *Aeromonas* bacterial isolates that are able to form biofilm showed resistance to multiple antibiotics compared to bacterial species that are not involved in the formation of biofilms (Hassan et al., 2011).Thus, a large number of isolates resistance to multiple antibiotics in this study could be due to their ability to form biofilms within drinking water containers. Gene transfer among *Aeromonas* spp. biofilms bacterial population in household's storage containers contribute to the high resistance of bacteria in drinking water (Molin and Tolker-Nielsen, 2003).



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Furthermore, many strains of *A. hydrophila* have been reported to harbour mobile elements that encode antibiotic resistance and can be transferred among themselves or to other bacterial species to establish multiple antibiotic resistances (Al-Fatlawy and Al-Hadrawy, 2014).

Data suggest that biofilms play an important role in exchange of genes among *Enterobacteriaceae* bacterial species due to its structure that promotes horizontal gene transfer, especially by conjugation process (Molin and Tolker-Nielsen., 2003). It has been indicated in literature that antibiotic resistance of *Aeromonas* isolates from aquatic environment is principally plasmid mediated, and thus it is easily transferred among environmental strains (Rhodes et al., 2000). Microbial communities within biofilms have been microscopically observed to share nutrients, metabolites and genetic elements (Lopez et al., 2010). Thus, they are also able to share antibiotic resistance genes. Therefore biofilms within household's drinking water storage containers might play a role in the microbial contamination of drinking water and transmission of antibiotic resistant genes among bacterial species within same container. As a result, resistance genes can be transferred to humans and possibly their virulence factors to humans through consumption of such stored drinking water.

**Table 4.8:** Number of bulk water and biofilm isolates observed with multidrug resistance phenotypic profiles

| *No. of antibiotics* | *No. of Isolates that showed resistance to multiple antibiotics* | |
|---|---|---|
| | *Water isolates (n=12)* | *Biofilm isolates (n=18)* |
| *4* | 2 (17%) | 0 (0%) |
| *5* | 2 (8%) | 4 (22%) |
| *6* | 6 (50%) | 4 (22%) |
| *7* | 2 (25%) | 7 (39%) |
| *8* | 0 (0%) | 2 (11%) |
| *9* | 0 (0%) | 1 (6%) |

Antibiotic resistance profiles observed in this study clearly indicates that *Aeromonas* species from household drinking water are the reservoir responsible for dissemination of antibiotic resistance in drinking water. Similarly, dissemination of some of antibiotic resistance genes between environmental sources and pathogenic bacteria has been demonstrated (Wright, 2010). In agreement to this, data from a study done in Portugal on wastewater and drinking water suggest that *Aeromonas* isolates were the relevant reservoir responsible for the dissemination of antibiotic resistance in aquatic environment (Figueira et al., 2011).



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Furthermore, *Aeromonas* spp. have been observed to play a major role as a reservoir and vector of antibiotic resistance genes as well as plasmids (Goni-Urriza et al., 2000).

Resistance profiles from river water isolates showed a large number of resistance patterns than the ones from the tap water source. Therefore there is a high possibility that these antibiotic resistance genes to be passed on from pathogenic bacteria to humans through environmental contamination which include drinking water drawn from contaminated surface or ground water source. This is of health risk, given the fact that when the resistant bacteria enter humans, they have the opportunity to spread their antibiotic resistance genes to the human body (Wellington et al., 2013).

Several studies from other countries also showed multiple resistance profiles among *Aeromonas* isolates from drinking water from different sources. In India, all strains isolated from water sources regularly used for domestic purposes were multidrug resistant (Bhowmilk et al., 2009). Multiple antibiotic resistance profiles among *Aeromonas* isolates was also reported in treated and untreated drinking water in Lebanon (Tokajian and Haswa, 2004). In a study done in Eastern Cape Province of South Africa, *Aeromonas* spp. isolates from waste water treatment plants were observed with multiple resistant profiles to beta-lactams, tetracycline and trimethoprim (Igbinosa and Okoh, 2012).

**Table 4.9:** Number of isolates recovered from both bulk water and biofilm of the same storage households' container.

| Number of households storage container | Bulk water isolates | Biofilm isolates | Bacterial species identified |
|---|---|---|---|
| *1* | 17B | 17C | *Aeromonas media* |
| *2* | 55A | 55B | *Aeromonas hydrophila* |
| *3* | 57A | 57B | *Aeromonas hydrophila* |
| *4* | 92B | 92C | *Aeromonas hydrophila* |
| *5* | 95B | 95C | *Aeromonas hydrophila* |
| *6* | 98B | 98C | *Aeromonas caviae* |
| *7* | 100B | 100C | *Aeromonas hydrophila* |

Similar *Aeromonas* strains were identified in bulk water and biofilm isolates collected from a number of the same household drinking water storage containers as illustrated in Table 4.9. This is believed to have contributed to the exchange of antibiotic resistance genes from biofilms to the bulk water within the same storage container. Since bacteria in biofilms are known to be kept together by a self-produced biopolymer matrix that contains polysaccharides, proteins and DNA originating from attached to biofilm (Høibya et al., 2010). This emphasizes the fact that genetic material is easily exchanged at the sites of biofilms because of the



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closeness of the bacterial cells hence maintaining a large gene pool within the same environment (Paraje, 2011). Furthermore, biofilms are believed to provide bacteria with resistance to antimicrobial agents (Lynch et al., 2002) and therefore their presence in household drinking water containers contribute to increase in antibiotic resistance of associated bacteria.



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**Table 4.10:** A summary of bulk water and biofilm isolates confirmed as *Aeromonas* by 16S rRNA sequencing, their haemolytic activity, virulence factors and antibiotic phenotypes results.

| Isolates | Source | 16S rRNA Sequencing ID | Haemolysin Production | Virulence genes detected | | | | Antibiotic phenotype |
|---|---|---|---|---|---|---|---|---|
| | | | | asa1 | cylA | gelE | hyl | |
| **Bulk Water** | | | | | | | | |
| 17B | River | *Aeromonas media* | α haemolysis | - | - | - | - | AP-A-C-E-KF-OT |
| 55A | River | *Aeromonas hydrophila* | α haemolysis | - | - | - | - | AP-A-C-E-KF-OT |
| 57B | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF-OT |
| 61B | Tap | *Aeromonas hydrophila* | α haemolysis | - | - | - | - | AP-A-E-KF |
| 72B | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-E-KF-OT-TM |
| 87A | Tap | *Aeromonas hydrophila* | α haemolysis | - | - | - | - | AP-A-E-KF-OT |
| 92B | River | *Aeromonas media* | α haemolysis | - | - | - | - | AP-A-E-K-OT-TM |
| 95B | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-E-KF-OT-TM-S |
| 97B | Tap | *Aeromonas hydrophila* | α haemolysis | - | - | - | - | AP-A-E-OT |
| 98B | Tap | *Aeromonas caviae* | α haemolysis | - | - | + | - | AP-A-C-E-KF-OT |
| 99B | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF |
| 100B | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-KF-OT |
| **Biofilm** | | | | | | | | |
| 6C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF-OT-TM-S |
| 7C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF-OT-TM |
| 17C | River | *Aeromonas media* | α haemolysis | - | - | - | - | AP-A-C-E-KF-OT |
| 19C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF-TM |
| 53C | Tap | *Aeromonas hydrophila* | β haemolysis | - | - | + | - | AP-A-C-E-KF |
| 55C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-E-KF-OT-TM |
| 57C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF-OT |
| 58C | River | *Aeromonas media* | α haemolysis | - | - | - | - | AP-A-C-E- K-OT-S |
| 59C | River | *Aeromonas hydrophila* | β haemolysis | - | - | + | - | AP-A-E-KF-OT-S |
| 66C | River | *Aeromonas hydrophila* | α haemolysis | + | - | + | - | AP-A-E-KF-OT |
| 74C | Tap | *Aeromonas hydrophila* | α haemolysis | + | - | + | - | AP-A-E-KF-OT |
| 80C | Tap | *Aeromonas hydrophila* | α haemolysis | - | - | - | - | AP-A-C-E-K-OT-TM |
| 86C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF-OT |
| 92C | River | *Aeromonas media* | α haemolysis | - | - | - | - | AP-A-C-E-KF-OT-TM |
| 95C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-KF-OT-TM |
| 98C | Tap | *Aeromonas caviae* | β haemolysis | - | - | + | - | AP-A-C-E-KF-OT |
| 99C | River | *Aeromonas media* | β haemolysis | - | - | + | - | AP-A-C-E-K-KF-OT |
| 100C | River | *Aeromonas hydrophila* | β haemolysis | + | - | + | - | AP-A-C-E-K-KF-OT |

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## 4.8. Summary of data obtained in this present study

Although all bulk water and biofilm samples examined in this study displayed *Aeromonas* presumptive positive green colonies on *Aeromonas* selective agar through membrane filtration isolation technique; not all were identified as *Aeromonas* species by biochemical (API 20E) and molecular (16S RNA sequencing) identification techniques. Only 44% and 45% bulk water and biofilm isolates respectively were identified as *Aeromonas* spp. in accordance to the API 20E technique. A total of 38% samples identified as *Aeromonas* spp. by biochemical API 20E technique were confirmed by 16S RNA sequencing as species within the genus *Aeromonas*. Out of the 38% confirmed by 16S RNA sequencing, 15% were bulk water isolates and 23% were biofilm isolates.

Number of biochemically identified *Aeromonas* positive isolates that were not confirmed as members of the genus *Aeromonas* in accordance with the molecular identification technique were identified as commonly known opportunistic bacterial species that belonged to the family Enterobacteriaceae. Identified spp. include *Enterobacter* spp. (40%), *Klebsiella* spp. (10%), *Pseudomonas* spp. (5%), *Morganella morganii* (1%) and *Raoultella ornithinolytica* (1%). Other opportunistic bacterial spp. such as *Alcaligenes* (1%) and *Acinetobacter* (3%) were also identified. This is of health concern as members of the family Enterobacteriaceae are considered to be opportunistic bacterial species responsible for several human infections upon contamination in drinking water used for domestic purposes.

According to the study questionnaire data taken, 76% of households' members use raw water from local rivers as their alternative water source for bathing and other households' domestic purposes and majority use such water without application of any treatment before use. This might expose the water users to the water related bacterial species, including *Aeromonas* spp. as they occur naturally in untreated water sources (Ghenghesh et al., 2008). As a result, this present a health threat, especially in regard to the prevalent of 39% of immunocompromised individuals in such households as they are the most vulnerable group to *Aeromonas* associated infections. Literature have indicated that, diarrhoea infections due to *Aeromonas* spp. may last for a long period of time, particularly in the immunocompromised individuals (Obi and Bessong, 2002, USEPA, 2006). In addition to diarrhoeal infections, skin and soft tissues infections may develop in people with wounds, or open skin cut as a result of bathing with such untreated water.



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Identification of *Aeromonas* spp. from biofilms collected from households' drinking water storage containers support the findings that biofilms in drinking water are reservoirs of microbial contamination (Szewzyk et al, 2000; Wingender and Flemming, 2011). Poor sanitation and hygiene is thought to have contributed to high prevalence of *Aeromonas* spp. detected in this study. Majority of the households' drinking water storage containers in the study areas visited were not regularly washed and some of the containers had no lids. In addition, the microbiological quality of drinking water might have been deteriorated during long period of storage within households' storage containers that contribute to the formation of biofilms within containers. These in turn supports the regrowth of bacteria within households' drinking water. Therefore, the drinking water in the present study might be implicated in waterborne infections as its microbiological quality seems to be compromised.

The recovery of *Aeromonas* strains from biofilm samples could also contribute to resistance features of such biofilm associated strains to commonly used antibiotics and persistent infections in humans. According to literature, biofilm *Aeromonas* isolates acquire protective characteristic against antibiotic activity and host immune system. It is further reported that biofilm producing bacterial strains display higher levels of resistance to all classes of antibiotics; and in comparison to non-producing or non-attached biofilm *Aeromonas* strains are in the range of 10-2000 times more resistant (Thenmozhi et al., 2014). As a result, increase of *Aeromonas* spp. in resistance to multiple antibiotics poses added health risk factors.

The most predominant *Aeromonas* species in this study was *Aeromonas hydrophila* (73%), followed by *Aeromonas media* (20%) and *Aeromonas caviae* (7%). *Aeromonas hydrophila* was found as the predominant species in both bulk water and biofilm isolates. This data is consistent with the results reported by several authors, where *A. hydrophila* was the predominant species in different drinking water sources such as freshwater and municipal drinking water supplies (Ghenghesh et al., 2001; Koksal et al., 2007; Al-Sulami et al., 2014; Nashnoush et al., 2009; Oleiwi et al., 2014). Identified *Aeromonas species* has been proved to play a role in waterborne infections (Pablos et al., 2009; Khajanchi et al., 2010). *A. hydrophila* has been implicated in a variety of human infections mainly including gastroenteritis (Igbinosa et al., 2012), bacteraemia (Wu et al., 2011), septicaemia (Mahdi et al., 2008; Senderovich et al., 2012), followed by urinary tract infections (Ragunathan et al., 2012). These infections are usually treated with antibiotics, in humans, however, increasing frequency of occurrences of antimicrobial resistance have been reported (Janda and Abbott, 2010).



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Although this study has demonstrated high prevalent of *Aeromonas spp.*, research have indicated that infections to humans depend on the type of strain and type of virulence factor observed from isolates (Odeyemi and Ahmad 2013). In addition the detection of virulence factors in *Aeromonas spp.* is usually considered as a key element in the determination of potential pathogenicity because literature suggested that more than two virulence factors act multi-functionally and multi-factorially to cause infections in humans (Pablos et al., 2009). The data in the present study revealed production of two virulence gene factor from isolates. The production of haemolytic toxins was observed from isolates which is regarded as a strong evidence for the pathogenic potential of bacterial isolates.The production of more than one virulence gene factor, in combination with the haemolytic toxins indicates pathogenic potential of isolates, and this could exposes the consumers to health risk.

Moreover, the data in the present study also indicate the multiple antibiotic resistance strains of *Aeromonas* isolates. This is of health concern as multiple antibiotic resistance contribute to fewer therapeutically options. Increasing resistance to most commonly used antibiotics in treatment settings and also to multiple antibiotics poses added health risk to consumers. Ciprofloxacin was found to be effective against all isolates tested in this study, and thus can be used effectively for treatment of *Aeromonas* associated infections. Ciprofloxacin is a fluoroquinolone and widely used in the treatment of bacterial infectious diseases such as urinary, respiratory and gastrointestinal tract infections among other infectious diseases.

The large number of resistant isolates from untreated river water storage container were observed. This could be due to inappropriate use of antibiotics that is usually blamed for environmental contamination of natural water sources with antibiotics that contributes to the maintenance and spread of antibiotic resistance genes (Ghenghesh et al., 2008; Huddleston et al., 2008). As a result, this leads to increase in antibiotic resistant strains in environmental water including river water (Adesoji et al., 2014). Another explanation could be due to the production of chromosomally encoded beta-lactamases usually associated with penicillins and first generation cephalosporins (Janda and Abbott, 2010). Furthermore, *Aeromonas* species have been shown to possess integrons, which are believed to be responsible for the resistance to commonly used antibiotics including tetracyclines, aminoglycosides, chloramphenicol and trimethoprim (Carvalho et al., 2012).

Unidentified isolates by API 20E were identified by 16S rRNA sequencing and this means that 16S rRNA is sensitive and reliable for the identification of any unknown bacteria compared to the biochemical method of identification. The 16S rRNA gene sequencing identified each isolate to one bacterial species level. The 16S rDNA gene sequencing technique has been



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proved to be a valuable tool over the phenotypic and biochemical identification techniques for the identification of *Aeromonas* species (Wang et al., 2003). The 16S rRNA gene is present in all bacteria and it is not associated with extensive mutations and is thus reliable as an identification method (Clarridge, 2004). However phenotypic and biochemical characteristic of bacteria can change due to environmental conditions, temperature, level of pH and required substrate for growth (Janda and Abbott, 2002).

Therefore 16S rRNA gene sequencing is suggested to be more accurate that the phenotypic and the biochemical identification techniques, nonetheless it is not 100% accurate. 16S rRNA gene sequencing is sensitive and specific; therefore both Phenotypic and Genotypic identification method should be used for identification of bacterial species from environmental samples. Phenotypic identification techniques can always serve as the appropriate isolation technique for presumptive positive isolates, while genotypic identification techniques serve as the suitable confirmation tools for the presumptive positive isolates.



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# Chapter 5
# CONCLUSIONS AND RECOMMENDATIONS

## 5.1. Conclusions:

The aim of this study was to determine the prevalence, antibiotic resistance patterns and virulence capabilities of *Aeromonas* spp. from drinking water in storage containers from rural households in the Vhembe district, South Africa.

Four objectives were formulated to achieve the aim of this study as follows:

- **Isolation and purification of presumptive positive *Aeromonas* spp. from bulk water and biofilms samples using membrane filtration technique.**

*Aeromonas* spp. were successfully isolated on *Aeromonas* selective agar using membrane filtration technique. Furthermore, purification of these isolates was also a success by streak plating and Gram staining technique to differentiate the Gram-positive from Gram-negative as *Aeromonas* spp. are Gram-negative bacteria. Therefore, successful isolation of presumptive *Aeromonas* spp. from bulk water and biofilms samples served as microbial contamination indicator of health risk to the households' members as they rely on such drinking water for survival and other domestic purpose.

- **Identification of *Aeromonas* spp. using API 20E biochemical identification tests and 16S rRNA gene sequencing.**

Seventy three (44%) bulk water isolates and 45 (55%) biofilm isolates were identified as *Aeromonas* species by API 20E biochemical system. Species were identified as either *Aeromonas hydrophila/caviae/sobria* 1 or 2. The prevalence of *Aeromonas* species recovered in drinking water present a health risk to the members of households visited during the course of the present study.

*Aeromonas* spp. identified biochemically were confirmed through 16SrRNA sequencing in 15% bulk water isolates and 23% biofilm isolates. The 16S rRNA gene sequencing also successfully identified isolates that could not be identified with API 20E biochemical identification technique. Some *Aeromonas* species that were identified as either *A. hydrophila/caviae/sobria* according to API 20E were identified to single species level as *Aeromonas hydrophila, Aeromonas caviae and Aeromonas media* by 16S rRNA gene



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sequencing. *Aeromonas hydrophila* was identified as the most predominant strains amongst other *Aeromonas* strains identified from both bulk water and biofilm isolates. It is demonstrated in Chapter 4 that these results were in accordance with previous studies.

❖ **Haemolysin production and detection of virulence genes from isolates**

Seven (58%) of the 12 tested bulk water isolates and 12 (67%) of the 18 tested biofilm isolates displayed β-haemolysis. Therefore, *Aeromonas* strains isolated from bulk water and biofilms samples in this study might be involved with pathogenesis of associated infections. Since it is well known that the haemolytic activity that could be detected in bacterial isolates together with two or more virulence factor generally identifies the pathogenic potential of the bacterial strains.

Two virulence genes, namely gelatinase (*gelE*) and aggregation substances (*asa1)* were detected in both bulk water and biofilm isolates in the present study. Therefore, these isolates may be pathogenic if they get introduced into the host. Most A. *hydrophila* strains isolated from both bulk water and biofilm isolates has pathogenic potential as were usually detected with two virulence factor and β-haemolytic activity. The results suggest that there may be gene transfer amongst bacterial species in biofilms and those in bulk water within the same storage water containers. This is of health risk to consumers as these virulence factors can works together to cause gastrointestinal infections in humans. Therefore, the detection of virulence factors in *Aeromonas* species present their pathogenic properties and suggest a high risk of infection to the households' members in the study area. However, hyaluronidase (*hyl*) virulence gene and cytolysin (*cylA*) were not detected from both bulk water and biofilm isolates in this study.

❖ **Antibiotic susceptibility of haemolytic isolates**

A large number of the both bulk water and biofilm isolates were resistant to multiple antibiotics tested. The most frequently observed antibiotic phenotype was AP-A-C-E-KF-OT. The second most frequent antibiotic phenotype observed in this study was AP-A-C-E-K-KF-OT. Few individual isolates also showed resistant to TM and S. None of the *Aeromonas* isolates were resistant to CIP. It is possible that antibiotic resistance obtained from bulk water isolates was transferred from the biofilms through genetic elements. The high resistance and multidrug resistance patterns obtained in this study lessen the choice of antibiotics used for treatment infections caused by *Aeromonas* species. However, this data set can be useful in chemotherapy to select the most effective antibiotics for treatment of *Aeromonas* infections. Antibiogram phenotypic data in this study suggest that ciprofloxacin (CIP) can be used as a



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suitable antibiotic for the treatment of *Aeromonas*-associated infections, resulting from contact with water.

The data in this study clearly indicate that the drinking water stored for a longer period before use is possibly not microbial safe and may pose a health risk for contracting enteric diseases to the household members who rely on such water for consumption. Thus, it can be concluded in this study, that the high prevalence of *Aeromonas* species observed in this study suggest that the household drinking water can serve as a source of potential pathogenic bacterial species that can lead to the development of infections to humans or water consumers and reservoir of antibiotic resistant genes.

## 5.2. Recommendations:

The households' drinking water storage containers should be well maintained and cleaned properly by scrubbing the internal surfaces of the containers regularly before filling the containers with drinking water. In addition, if possible households' drinking water storage containers should be regularly disinfected in order to prevent the development of a biofilm on the inner wall of containers.

Sufficient time and enough residual chlorine level required to destroy the *Aeromonas* associated with biofilms should be determined in order to prevent regrowth of *Aeromonas* bacteria in drinking water storage containers, especially for water stored over a long period of time.

Routine monitoring for the prevalent of emerging waterborne bacteria such as *Aeromonas* spp. in drinking water, particularly in rural areas where they occasionally rely on untreated water sources should be considered.

Examination of the health risks associated with *Aeromonas* spp. is necessary to update the quality guidelines for drinking water due to the high prevalence of *Aeromonas* species in drinking water and several virulence genes detected.In addition, further studies should use a reliable complete set of virulence gene markers for *Aeromonas* strains responsible for human infections in order to determine pathogenicity potential of isolates.

The local governmental water and health authorities should make sure that the taps in rural areas of Vhembe district are properly functioning and water is always available. This will help to avoid long period of storage of municipal treated drinking water before use in rural areas of



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SA as this promotes the regrowth of *Aeromonas* bacterial species. However, in some rural areas, this might not be executable to long term as public services struggle to keep up on providing treated, piped water to people.

Therefore, in the meantime rural communities should be made aware on the importance of domestic water hygiene as well as storage container hygiene. Household members should also be educated to apply sustainable anti-microbial contamination measures during water collection, handling and usage during storage at home (Jagals et al., 2003; Nala et al., 2003).



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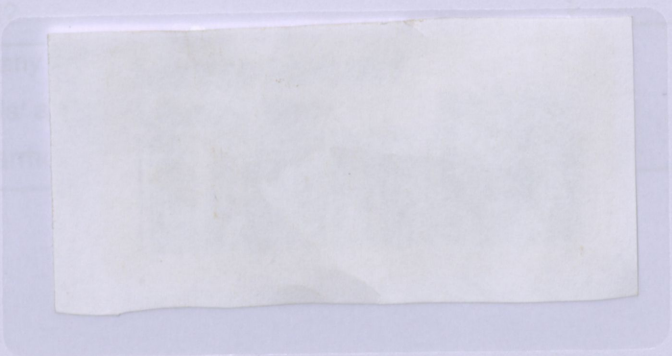
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# APPENDIXES

**Appendix 1:** Questionnaire template used for gathering information concerning drinking water samples collected in households' visited for this present study.

| Name of village: | DATE: | Stand NO: | SAMPLE NO: |
|---|---|---|---|
| | | | |

| | | |
|---|---|---|
| 1. | What is the source of available drinking water in use? | |
| 2. | Where do you usually fetch water for drinking? | |
| 3. | Is water always available in the above mentioned source? | |
| 4. | Do you have any other alternative source of water? | |
| 5. | Who normally goes and collects water? | |
| 6. | What kind of containers do you use to fetch water? | |
| 7. | What kind of transports do you use to fetch water? | |
| 8. | How long does it take from the source to the households? | |
| 9. | How long do you store the water before use? | |
| 10. | What do you do before you use the water for drinking, any kind of treatment before use? | |
| 11. | How many people depend on that water for the household? | |
| 12. | Are there any immunocompromised individuals in the households/ any member suffered from water related illness (diarrhoea)? | |



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**Appendix 2:** NCCLS used for measuring zones of inhibition around the antibiotics disk used for determination of antibiotic susceptibilities of bulk water and biofilm isolates (National Committee for Clinical Laboratory Standards (NCCLS).

| Antibiotics tested | Disk Code | Disk concentration (µg) | Diameters of Inhibition Zones (mm) | | |
|---|---|---|---|---|---|
| | | | Resistant | Moderate sensitive | Sensitive |
| 1. Ampicillin | AP | 10 µg | ≤13 | 14-16 | ≥17 |
| 2. Amoxicillin | A | 10 µg | ≤13 | 14-17 | ≥18 |
| 3. Cephalothin | KF | 30 µg | ≤14 | 15-17 | ≥18 |
| 4. Ciprofloxacin | CIP | 5 µg | ≤15 | 16-20 | ≥21 |
| 5. Chlorampenicol | C | 10 µg | ≤12 | 13-17 | ≥18 |
| 6. Erythromycin | E | 15 µg | ≤13 | 14-22 | ≥23 |
| 7. Kanamycin | K | 30 µg | ≤13 | 14-17 | ≥18 |
| 8. Oxytetracycline | OT | 30 µg | ≤11 | 12-14 | ≥15 |
| 9. Streptomycin | S | 10 µg | ≤11 | 12-14 | ≥15 |
| 10. Trimethoprim | TM | 2.5 µg | ≤10 | 11-15 | ≥16 |



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**Appendix 3:** Antibiotic susceptibilities shown in bulk water and biofilm samples from both (river and tap) drinking water sources

| Antibiotics | Concentration of antibiotics | Bulk water and biofilm samples from households storage containers (zone of inhibition diameter in mm value) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Bulk water samples (n=12) | | | Biofilm samples (n=18) | | |
| | | R | I | S | R | I | S |
| 1. Ampicillin | AP (10µg | 12 (100%) | 0 (0%) | 0 (0%) | 18 (100%) | 0 (0%) | 0 (0%) |
| 2. Amoxicillin | A (10µg) | 12 (100%) | 0 (0%) | 0 (0%) | 18 (100% | 0 (0%) | 0 (0%) |
| 3. Cephalothin | KF (30µg) | 10 (83%) | 2 (17%) | 0 (0%) | 16 (89%) | 2 (11%) | 0 (0%) |
| 4. Ciprofloxacin | CIP (5µg) | 0 (0%) | 0 (0%) | 12 (100%) | 0 (0%) | 1 (6%) | 17 (94%) |
| 5. Chloramphenicol | C(10µg) | 6 (50%) | 4 (33%) | 2 (17%) | 14 (78%) | 3 (17%) | 1 (5%) |
| 6. Erythromycin | E(15µg) | 12 (100%) | 0 (0%) | 0 (0%) | 18 (100%) | 0 (0%) | 0 (0%) |
| 7. Kanamycin | K(30µg) | 3 (25%) | 7 (58%) | 2 (17%) | 10 (55%) | 5 (28%) | 3 (17%) |
| 8. Streptomycin | S (10µg) | 1 (8%) | 3 (25%) | 8 (67%) | 3 (17%) | 4 (22%) | 11 (61%) |
| 9. Oxytetracycline | OT (30µg) | 10 (83%) | 2 (17%) | 0 (0%) | 16 (89%) | 2 (11%) | 0 (0%) |
| 10. Trimethoprim | TM (2.5µg) | 2 (17%) | 6 (50%) | 4 (33%) | 7 (39%) | 8 (44)% | 3 (17%) |

R=Resistant; I=Moderate sensitive; S=Sensitive

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**Appendix 4**: Comparison of antibiotic susceptibilities shown amongst bulk water isolates from river and tap source household stored water for drinking water

| Antibiotics | Concentration of antibiotics | Bulk water samples from households storage containers (zone of inhibition diameter in mm value) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Source : river water ( n=8) | | | Source: tap water (n=4) | | |
| | | R | I | S | R | I | S |
| 1. Ampicillin | AP (10µg | 8 (100%) | 0 (0%) | 0 (0%) | 4 (100%) | 0 (0%) | 0 (0%) |
| 2. Amoxicillin | A (10µg) | 8 (100%) | 0 (0%) | 0 (0%) | 4 (100%) | 0 (0%) | 0 (0%) |
| 3. Cephalothin | KF (30µg) | 7 (88%) | 1 (12%) | 0 (0%) | 3 (75%) | 1 (25%) | 0 (0%) |
| 4. Ciprofloxacin | CIP (5µg) | 0 (0%) | 0 (0%) | 8 (100 %) | 0 (0%) | 0 (0%) | 4 (100%) |
| 5. Chloramphenicol | C(10µg) | 5 (63%) | 3 (37%) | 0 (0%) | 1 (25%) | 1 (25%) | 2 (50%) |
| 6. Erythromycin | E(15µg) | 8 (100%) | 0 (0%) | 0 (0%) | 8 (100%) | 0 (0%) | 0 (0%) |
| 7. Kanamycin | K(30µg) | 3 (37%) | 4 (50%) | 1 (13%) | 0 (0%) | 3 (75%) | 1 (25%) |
| 8. Streptomycin | S (10µg) | 1 (13%) | 3 (37%) | 4 (50%) | 0 (0%) | 0 (0%) | 4 (100%) |
| 9. Oxytetracycline | OT (30µg) | 7 (87%) | 1 (13%) | 0 (0%) | 3 (75%) | 1 (25%) | 0 (0%) |
| 10. Trimethoprim | TM (2.5µg) | 2 (25%) | 4 (50%) | 2 (25%) | 0 (0%) | 2 (50)% | 2 (50%) |

R=Resistant; I=Moderate sensitive; S=Sensitive

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**Appendix 5:** Comparison of antibiotic susceptibilities shown amongst biofilm isolates from river and tap households drinking water storage containers

| Antibiotics | Concentration of antibiotics | Biofilm samples from households' storage containers (zone of inhibition diameter in mm value) | | | | | |
|---|---|---|---|---|---|---|---|
| | | source : river household storage (n=14) | | | source: tap households storage (n=4) | | |
| | | R | I | S | R | I | S |
| 1. Ampicillin | AP (10µg | 14 (100%) | 0 (0%) | 0 (0%) | 4 (100%) | 0 (0%) | 0 (0%) |
| 2. Amoxicillin | A (10µg) | 14 (100%) | 0 (0%) | 0 (0%) | 4 (100%) | 0 (0%) | 0 (0%) |
| 3. Cephalothin | KF (30µg) | 13 (93%) | 1 (7%) | 0 (0%) | 3 (75%) | 1 (25%) | 0 (0%) |
| 4. Ciprofloxacin | CIP (5µg) | 0 (0%) | 1 (7%) | 13 (93%) | 0 (0%) | 0 (0%) | 4 (100%) |
| 5. Chloramphenicol | C(10µg) | 11 (79%) | 3 (21%) | 0 (0%) | 3 (75%) | 0 (0%) | 1 (25%) |
| 6. Erythromycin | E(15µg) | 14 (100%) | 0 (0%) | 0 (0%) | 4 (100%) | 0 (0%) | 0 (0%) |
| 7. Kanamycin | K(30µg) | 8 (57%) | 4 (29%) | 2 (14%) | 2 (50%) | 1 (25%) | 1 (25%) |
| 8. Streptomycin | S (10µg) | 3 (21%) | 3 (21%) | 8 (57%) | 0 (0%) | 1 (25%) | 3 (75%) |
| 9. Oxytetracycline | OT (30µg) | 13 (93%) | 1 (7%) | 0 (0%) | 3 (75%) | 1 (25%) | 0 (0%) |
| 10. Trimethoprim | TM (2.5µg) | 6 (43%) | 5 (36%) | 3 (21%) | 1(25%) | 3 (75)% | 0 (0%) |

R=Resistant; I=Moderate sensitive; S=Sensitive

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