DETERMINATION OF *LEGIONELLA SPP* PREVALENCE IN HOT SPRINGS, LAKES, HOSPITALS AND DRINKING WATER DISTRIBUTION SYSTEMS IN ETHIOPIA

BY

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This is to certify that the thesis prepared by Thewodros Bekele Tolera, entitled: *Determination of Legionella Spp Prevalence in Hot Springs, Lakes, Hospitals and Drinking Water Distribution Systems in Ethiopia* submitted in partial fulfillment of the requirements for the degree of Doctor of philosophy (Environmental Science) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Determination of Legionella Spp Prevalence in Hot Springs, Lakes, Hospitals and Drinking Water Distribution Systems

Thewodros Bekele

Addis Ababa University, 2020

Legionnaires' disease is an emerging atypical pneumonia type of illness caused by inhalation of aerosols contaminated by Legionella Spp. There is limited information about the prevalence of Legionella Spp., from Ethiopian water systems. A cross-sectional descriptive study which entails the quantitative investigation of Legionella spp from Hot Springs, Lakes, hospitals and drinking water distribution Systems was conducted from Dec 14, 2016, to June 20, 2018. Representative water and biofilm samples collected and analyzed following standard procedures. 1L of water samples was concentrated by membrane filtration techniques by using 47 mm diameter cellulose membranes with a pore size of 0.2μm, microbes retained by the filter transferred to a sterile test tube containing distilled water and further eluted from the filter by vortex-mixing for 5-10 minutes. Biofilm samples were collected from water distribution system by sterilized Dacron Tipped swabs by rubbing three to four times inside water pipe. For Legionella isolation, about 0.1 ml of filtered and biofilm samples were spread plated on Buffered Charcoal Yeast Extract Agar, while Plate count agar were used for Heterotrophic plate Count and standard microbial procedures were followed. physicochemical qualities of water samples analyzed by portable digital photometer instruments following instrument instructions. Among a total of 220 water samples 46(43.3%) of hot spring samples; 22(38.6%) of hospital water samples; 8(30.7%) of Addis Ababa water distribution system and 19(61.3%) of natural water samples were positive to Legionella spp. The Legionella colonies of hot springs range from Log_{10} 2.60 ±0.69 to 3.22±0.78; natural water bodies 1.66±0.8 to 3.29±0.3; Addis Ababa water distribution samples 1 to 1.41±0.8 and Hospital water samples 1.22±0.46 to 2.8±1.41 Log_{10} cfu/l. Meanwhile 23(21.7%), 12(21.05%) and 3(11.5%) of hot springs, hospital water samples and Addis Ababa water distribution system had HPC >300cfu/ml respectively, while 23(74.2%) natural water bodies had HPC>2500cfu/ml. Temperature (^{O}C) range were 35.3±1.4 to 48.3±5.5 in hot springs

while from 18.6±1.1-25.2±0.4 in natural water bodies; pH varies 6.5-7.5 in hot springs; 6.9-7.4 in hospital water; 6.6 to 8.5 in natural water bodies; mean Fluoride and Iron levels range 2.6-30.4 and 6.1-23.1mg/l in hot springs; 0.2-0.4 and 0.6-0.6mg/l in hospital water samples; 0.2-7.1 and 0.4-8.4mg/l in natural water bodies but range from 0.21-30.4 and 0.34-23.1mg/l respectively. The mean free residual chlorine from the sampled hospital and Addis Ababa water distribution system was below 0.2mg/l. The minimum and maximum concentration of elements all samples ranged, Potassium 1.9-44.8mg/L; Phosphate 4.95-48.3mg/L; Sulfate 2.2-59.7mg/L; Total alkalinity 61.7-4752.0mg/L; Total Dissolved Solids 88.7-2569mg/L; Electrical Conductivity 130-1813.7(µS/cm). Significant level of correlation was observed between microbial parameters of Legionella and heterotrophic plate count. HPC correlated with Legionella at P<0.05(r=-0.494) HS2; (r=0.464) Yekatit 12 hospital but at P<0.01 (r=-0.528) HS1; (r=-0.722) HS3; (r=0.859) AAWDS; (r=-0.461) Zewditu hospital (ZH). Significant positive correlations observed between Legionella and Temperature r= 0.424(HS2), with TDS r=0.463(HS1), with phosphate r=0.497(HS4); whereas negative correlations observed with fluoride r=-0.497(HS4), Temperature r =-0.416(Y12H), with free residual chlorine between r=-0.468 to -0.751 from all of sampled hospitals and Addis Ababa water distribution system. This study indicates the presence of Legionella within diverse physicochemical water sources and can enlarge our knowledge towards Legionella and associated physical-chemical and microbial parameters from Ethiopian water bodies. Education and awareness have to be given for potable water suppliers, property or building owners about risks associated with Legionella and other infections. Potable water systems should have to maintain adequate treatment residual chlorine and practice good pipe maintenance as important water safety plans. Water systems have to be closely monitored the microbial level and mechanisms to control and prevent possible outbreak are needed by owners, stakeholders, and regulatory bodies. Legionella species identification by molecular methods needs to be determined by further study.

Keywords: *Legionella Spp, HPC, Physicochemical and Microbiological quality, Water Distribution System, Hot springs, Hospitals, Lakes, River*

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Thewodros Bekele Tolera

AAU, Ethiopia

To My Family: DAD (Bekele Tolera), MOM (Alemush Adugna) and My WIFE (Hannu) and KIDS Selam, Maria and Nigus

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LIST OF ACRONYMS

AAWDS	Addis Ababa Water Distribution System
AAWSA	Addis Ababa Water & Sewerage Authority
APHA	American Public Health Association
AWT	American Water Technology
ART	Antiretroviral viral therapy
BAL	Broncho Alveolar Lavage
BCYEA	Buffer Charcoal Yeast Extract Agar
BGS	Biritish Geological Survey
CAP	Community-Acquired Pneumonia
CDC	Centers for Diseases Control and prevention US
CES	Compulsory Ethiopian Standards
CFU	Colony Forming Unit
COPD	Chronic Obstructive Pulmonary Disease
CSA	Central Statistical Agency
DFA	Direct Fluorescent-Antibody
DHS	Demographic and Health Survey
DNA	Deoxyribonucleic acid
DOT	Defective Organell Trafficking
DWDS	Drinking Water Distribution System
EC	Electrical Conductivity
ECDC	European Centre for Disease Prevention and Control

EIA	Enzyme Immuno Assay
EIDs	Emerging infectious diseases
ELISA	Enzyme-Linked Immuno Sorbent Assay
EM	Environmental Mycobacteria
EMA	Ethidium MonoAzide bromide
EPA	Environmental Protection Agency (US)
EU	European Union
FLA	Free Living Amoeba
GVPC	Glycine Vancomycine Polymyxin Cycloheximide
HAI	Health care Associated Infections
HAP	Hospital-Acquired Pneumonia
HPC	heterotrophic Plate Count
HPSC	Health Protection Surveillance Center
HSP	Heat Shock Protein
ICM	Interacellular Multiplication gen
ICT	Immuno Chromogenic Test
IFA	Indirect immune Fluorescence Assay
ISO	International Standardization organization
LD	Legionnaires' Disease
LLAPs	Legionella-Like Amoebal Pathogens
LP	*Legionella pneumophila*
MDWSTs	Municipal Drinking Water Storage Tanks
MIP	Macrophage Infectivity Potentioator protein

MOH	Ministry of Health
MOMP	Major Outer Membrane Protein
MPL	Maximum Permissible Level
OPPPS	Opportunistic Premise Plumbing Pathogens
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
qPCR	Quantitative real time PCR
RVL	Rift Valley Lakes
SDG	Sustainable Development Goal
TALD	Travel-Associated Legionnaires' disease
TDS	Total Dissolved Solids
UAT	Urinary Antigen Test
UNAM	University of Namibia
UV	Ultra Violet light
VBNC	Viable But Not Culture able
WHO	World Health Organization
WSP	Water Safety Plan
Y12H	Yekatit 12 Hospital
ZH	Zewditu Hospital

CHAPTER ONE

1. INTRODUCTION

1.1 Background

Societal development result alterations of human lifestyle, natural environment and lead to development or civilization associated health pros and cons. In fact, the enabling environment that we have created for various diseases and their effect that it has on our lifestyle are less well known. But some emerging infectious diseases like Legionellosis and others are reported in connection with urbanization and industrialization (Kozioł *et al*., 2014; WHO, 2012).

Emerging infectious diseases (EIDs) are diseases of infectious origin whose incidence in humans has increased within the recent past or threatens to increase in the near future. The prevalence of new, previously undefined diseases as well as old diseases with new features is also included in EIDs. For instance, the introduction of a disease to a new location or a new population (e.g. it may present in youth where previously it was only seen in the elderly); new clinical features, including resistance to available treatments; or a rapid increase in the incidence and spread of the disease. Reappearance of a disease which was once endemic but had since been eradicated or controlled would be classified as a re-emerging infectious disease. Emergence may also be due to a new recognition of an infectious agent in the population or the realization that an established condition has an infectious origin. In the last four decades, over 30 new infectious agents have been detected worldwide including Legionellosis, Lyme disease, and others (Kozioł *et al*., 2014; Dikid *et al*: 2013).

Legionellosis is a collection of infections caused by *Legionella pneumophila* (*Lp)* and related bacteria are causing Legionellosis which is atypical pneumonia that can be sporadic or epidemic, and community acquired or nosocomial pneumonia (Phares *et al,* 2007). Water is an important medium for many pathogenic organisms, causing a high disease burden on global health. Consequently, water-borne pathogen contamination in water resources and related diseases are a water quality concern throughout the world (Pandey *et al.*, 2014).

The provision of piped water directly to the household has been associated with improved hygiene and reduction in disease. However, as standards of living have risen and water infrastructures have aged, there has been growing recognition that water distribution systems are vulnerable to intrusion, contamination and may contribute to endemic and epidemic waterborne disease. In this connection, Legionnaires' disease (LD) is a waterborne disease often associated with man-made water systems and is considered as an emerging public health problem and is linked to high rates of mortality and morbidity, if not properly treated. LD was first discovered in Pontiac, Michigan, USA in 1968 but the etiological agent was not known. but, in 1976 LD gets limelight when an outbreak of community pneumonia called Legionnaires' disease (LD) occurred at USA, Philadelphia Legion with its causative agent *Legionella pneumophila* identified (Alexis & Mark , 2018). Humans who have weak immune system and the elderly are highly susceptible for *Legionella* infection. Infection occur when someone inhale aerosol contaminated by *Legionella* bacteria (Kozioł *et al*., 2014; Gordon, 2007 and Bartram *et al.*, 2006).

Worldwide the causative agent for LD traced to a wide variety of environmental water sources such as drinking water system, cooling towers, hot tubs, showerheads, whirlpools and spas, and public fountains (Atlas, 1999; Doleans *et al*., 2004). *Legionella Spp* can be isolated from

drinking water biofilms (Lu *et al*., 2014) and can colonize hot and cold water supply system in large public buildings, households, and industrial systems. *L. pneumophila* thrives within the biofilm of premise plumbing systems, utilizing protozoan hosts for protection from disinfectants and other environmental stressors (Alexis & Mark, 2018). Different factors are contributing for the colonization of *Legionella Spp* in manmade or artificial water distribution systems. To mention some, increased temperature (optimal temperature 20°C-45°C), the presence of sludge, mud, corrosion products, biofilm, and biological agents (other bacteria, protozoa), the stagnation of water installation (no recirculation, "dead legs" of the installation), and too low concentration of the disinfectant (Gordon, 2007; Bartram *et al.*, 2006; Kozioł *et al*., 2014).

To this end water distribution system, especially drinking water distribution systems (DWDS) may create conducive environment for pathogenic microbial growth in the system resulting waterborne infections if they are not properly monitored and maintained. Moe and Rheingans (2006) reported an increasing trend of waterborne disease outbreaks in USA to be associated with problems in water distribution systems. For instance, *Legionella pneumophila* alone accounts for some 30% of U.S. drinking water-related outbreaks and about 80% of drinking water-related deaths in the United States (Lu *et al*., 2014). Specifically, about three fold growth rate cases of Legionnaires' disease from water distribution system in the USA were reported by CDC with in nine years from 1110 in 2000 to 3522 in 2009 (CDC,2011).The incidence rate increased from 0.39 to 1.15 per 100 000 people during that time.

Biofilm formation in water distribution system can provide a means for survival and dissemination of microbes including *L. pneumophila*, and interfering with efforts to eradicate bacteria and others from water systems. Additionally, a number of factors the nature/level of

pipe surface materials, concentration, quality of nutrients and disinfectants, available temperature, hydraulics of the system and others physic- chemical factors have their own role for the biofilm formation and accumulation of microbes with in water distribution system (Murga *et al*., 2001; Zachev *et al*., 2001; Lin *et al*., 1998; Momba *et al*., 2000; Zacheus and Martikainen, 1996).

An outbreak of LD is a growing concern in developed countries due to the reporting or diagnosing mechanism they have, or a rise in susceptible groups for infection and other environmental conditions. Amplification of *Legionella* concentrations within building plumbing systems is a major contributor to increased risk, but distribution systems can represent the source of the original inoculums (WHO, 2014). Despite, conditions for *Legionella* infections are available in the world; most LD infection has been documented from developed nations. Accurate estimates of the incidence of specific pneumonia etiologies are valuable for appropriate allocation of public health resources, establishing vaccine priorities, and developing evidence-based standards for treatment (Phares *et al*, 2007).

Some limited case studies from Africa revealed the presence of *Legionella spp* from the natural and manmade environment. For instance, Chinsembu *et al*. (2010) isolated *L. pneumophila* from Goreangab Dam water and student hostel shower heads in Namibia; Ali *et al*., (2011) reported prevalence of *Legionella* (8%) in streams and 1% in wells from Nigeria. Moreover, *Legionella* prevalence was reported from water distribution systems in Morocco by Mariam *et al.,* (2012), from Namibia by Chinsembu *et al*., (2010); from South Africa by Singh and Coogan,(2005) and Gabon Ehrhard *et al*.,(2015).

Legionella infection was reported from African pneumonia patients relatively higher up to 22.7% from Sudan with highest prevalence 36.8% found from patients of (31-45 years) age

group (Rabih, *et al*., 2014) and relatively lower level of infection was reported as 9.2% in Kenya (Odera and Anzala, 2009), 5% from Egypt (Goda *et al*., 2009) and 1.2% from South Africa (Nicole *et al.*, 2016).

1.2 Statement of the Problem

Legionellosis is an emerging environmental issue that causes atypical pneumonia. The fundamental environmental and human factors are available in Africa but lacks accurate information about *Legionella Spp* prevalence and infection. Accurate estimates of the *Legionella* related information's are valuable for appropriate allocation of public health resources, treatments, awareness and developing evidence-based standards for treatment (Phares *et al.*, 2007). Actually, the true incidence of LD varies widely according to the setting investigated and the diagnostic methodology applied. Since many developing countries lack appropriate methods of diagnosing of *Legionella* infection or surveillance systems capable of monitoring the situation, the real magnitude of the problem is unknown and it may be responsible for more of the pneumonia occurring in the tropics than is generally recognized (Odera and Anzala, 2009).

Despite the fact that, environmental *Legionella* monitoring is recommended in several countries, monitoring of *Legionella Spp* is not conducted in Ethiopia and no active surveillance program exists and so prevalence of Legionnaires' disease and associated cases are unavailable. Ministry of Health (MoH) presented pneumonia as one of top ten causes of morbidity, where 16% and 8% are affected among <5 years children and >5 years and adults, respectively (MoH, 2012). Among other risk factors, immunocompromization is an important factor for development of *Legionella*-caused pneumonia. In line with this, immunecompromization due to HIV (with current prevalence of 0.9% within age group 15-

49 and its occurrence being seven times higher in urban (2.9%) areas than in rural (0.4%) areas (CSA, 2016). Report from WHO (2018) indicates harmful consumption of alcohol and tobacco showing increasing trends. Increasing trend of non- communicable diseases like Diabetes, Cancer, and others are indicating the presence of immunocompromised people immunosuppressed people in Ethiopia subjected to emerging waterborne infections (MoH, 2007; Help Age International, 2013; Abebe, *et al*., 2013).

Due to the rise of economic activity in Ethiopia, urbanization is expanding and averaged life expectancy increased from 45 years in 1990 to 64.8 years in 2016 (WHO, 2018). On the other hand, lack of safe water distribution in urban areas is a problem. In most towns water distributions lines are getting old; water line leakages are rampant and there is lack of water safety regulations in health care facilities, recreational areas which are prone to microbial contamination. With people getting old and thus immune-compromised they become susceptible to opportunistic infections such as *Legionella* infection. This indicates the presence of both environmental and human factors for the *Legionella* growth, multiplication and infection in water distribution systems. Hence *Legionella* might have been spread among the community and hospital distribution water systems.

In Ethiopia, there is no documented information regarding the prevalence of *Legionella spp* and others opportunistic waterborne infections from natural water bodies, hot springs, drinking water distribution systems. Generally, studies on the physicochemical and biological composition of surface water bodies such as Lakes are still scarce and limited (Rim, 2013). Particularly, isolation and detection of *Legionella spp* from Ethiopian water environment is lacking. Ecotourism is expanding around hot springs Sisay *et al*., (2015a) and around Lakes. Susceptible groups are usually visiting such hot springs and recreational water facilities. With the prevailing poor water quality management system in recreational sites Thewodros &

Seyoum, (2014) it is important to survey and closely monitor the presence opportunistic pathogens in hot springs and other natural water environments (Amanuel *et al*., 2017).

Meanwhile *Legionella spp* as opportunistic pathogen can be cultured by routine methodologies that involve the use of selective medium, periodic surveillance of water environment is required. Considering, the absence of such investigation in Ethiopia, it is important to initiate studies that explore *Legionella* from natural and manmade water environments that includes both hospital and non-hospital environments be started. Hence the present study focused on surveying *Legionella spp* from Ethiopian aquatic environment by taking samples from hot springs, natural Lakes, Dam and River and manmade water systems and the role associated factors in relation to water quality management on which the bacteria can grow and multiply.

The results and recommendations of this study will provide evidence based information to health, water and sanitation stakeholders so that on they would formulate appropriate pneumonia management and water and sanitation policies, respectively. The study will also contribute to science by adding information to the available knowledge pool about *Legionella*. Therefore, this is perhaps the first study of its kind aimed at investigating prevalence and frequency of *Legionella spp* from selected hot spring waters, natural water bodies and hospitals drinking water distribution system with the view to determine the prevalence of the species.

1.3 Research Objectives

1.3.1 General Objective

The general objective of the study was to investigate the prevalence of *Legionella Spp* and evaluation of the physicochemical and microbiological water quality association from selected Hot springs, natural water bodies and potable water distribution system in hospitals and Addis Ababa, Ethiopia

1.3.2 Specific Objectives

The specific objectives of this study were to:-

I. determine the prevalence of *Legionella Spp* from selected hospital's water distribution system in Addis Ababa, Ethiopia;

I. estimate the level of *Legionella Spp* colonization from selected Ethiopian hot spring spa water facilities;

II. enumerate *Legionella spp* from selected natural water bodies (Lakes and river) in Ethiopia;

III. Assess the level of *Legionella spp* colonization presence from drinking water distribution system in Addis Ababa, Ethiopia

IV. Associate the physicochemical water quality parameters with the presence of *Legionella spp and HPC.*

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Legionnaires Disease / Legionellosis / LD

2.1.1 History

Legionellosis is a collection of infections that emerged in the second half of the 20th century, caused by *Legionella pneumophila* and related *Legionella* bacteria (WHO, 2007). The disease limelight when 58th of American Legion convention was held (July, 1976) at Philadelphia, Bellevue Stratford Hotel. There were 4000 attendants at that time and of these 600 of them were staying at the hotel. Illness symptom began on the second day and on wards of those attending the convention, 221 became ill with pneumonia, and 34 of those affected died. In January 1977, Joseph McDade of the U.S. Centers for Disease Control (CDC), discovered the etiologic bacterium for the unexplained pneumonia outbreak at the 1976 American Legion convention in Philadelphia. The aerobic gram-negative bacteria isolated from infected post-mortem lung tissue and identified as the causative agent of unexplained pneumonia outbreak was later called *Legionella pneumophila*, receiving the name *Legionella* to honor the stricken American legionnaires and pneumophila from the Greek word meaning "lung-loving" (USEPA 1999; WHO, 2007).

2.1.2 Microbiology

Legionella Spp are ubiquitous in the aqueous environment of all types and have been isolated from natural environments in different areas of the world. The bacteria do not grow on routine bacteriologic media, rather on Buffered Charcoal Yeast Extract agar (BCYE). Soluble iron and L-cysteine are required for optimal growth and for the initial isolation of the bacterium from both clinical and environmental sources. Hence, Iron, L-cysteine, α-ketoglutarate, and charcoal-containing yeast extract agar buffered with an organic buffer (BCYEα agar) is the preferred growth medium for clinical isolation. To support bacterial growth, the pH of the agar is critical and should be adjusted to pH 6.9 by adding N-2-acetamino2-aminoethansulfonic acid (ACES). *Legionnella spp*., are obligate aerobes, and grow and multiply at temperatures ranging from 20^{o}C to 42^{o}C. Clinically important *Legionella* species grow best at 35^{o}C in humidified air on BCYEα medium, usually in 2 to 5 days after inoculation of plates (Diederen, 2007, WHO, 2007).

2.1.2.1 Morphology

All *Legionella* species appear as Gram-negative coccobacilli. Elongated filamentous forms may be seen after growth on some culture media. Unlike most gram-negative bacteria, *Legionella* cell walls contain high amounts of branched chain cellular fatty acids and ubiquinones with side chains of 9-14 isoprene units that make cell staining difficult. *Legionella* are urease-negative, catalase-positive, heterotrophic, aerobic, chemo-organotrophic, and most of them transitionally motile (WHO, 2007). When motile, they have one or more straight or curved polar or lateral flagella. They are un-encapsulated, non- spore forming, with physical dimensions from 0.3 to 0.9 μm in width and from 2 to 20 μm in length.

According to Jalila *et al*., (2012) and Strickhouser (2007) *Legionella spp.* utilize amino acids for energy and carbon, do not oxidize or ferment carbohydrates, and require L-cysteine-HCl and iron salts for growth amongst other nutrients is based on the catabolism of amino acids for energy and carbon sources.

2.1.2.2 Taxonomy

Legionella s*pecies* are classified based on DNA analysis and antigenic analysis of various proteins and peptides. Recent studies using 16S rRNA analysis confirm that the family *Legionellaceae* is a single monophyletic subgroup within the gamma-2 subdivision of the Proteobacteria belonging to the γ-proteobacterial lineage (Chien *et al*. 2004).

Currently, *Legionella* genus includes more than 52 species with about 70 different sero-groups. However, the number of recognized species and sero-groups of the genus *Legionella* continues to increase. *Legionella Spp.,* isolation and diagnosis from clinical material indicated that higher than twenty (20) species have been proven as a causative agents of Legionnaires' disease. *L. pneumophila (Lp)* with its fifteen (15) different sero- groups is responsible for majority 90% of confirmed infection cases of Legionellosis. *L. pneumophila* sero-group1 is responsible for over 84% of cases worldwide. Studies provided strong evidence that *L. pneumophila* is more pathogenic to humans than other *Legionella* species (Mariam *et al*., 2013).

2.1.3 Ecology

Legionella Spp., can survive in varied water conditions, in temperatures of 0-68°C, a pH range of 5.0-8.5, and a dissolved oxygen concentration in water of 0.2-15ppm. With the exception of natural hot springs where temperature ranges from 35°C to 40°C, the sources of legionellosis are exclusively man-made water systems. In water, a temperature range between 20°C to 45°C favors the growth of *L. pneumophila*. At lower temperatures, *Legionella* appears to enter into a dormant stage until exposed to more favorable conditions. *Legionella* survival is enhanced by symbiotic relationships with other microorganisms; sediment within biofilms stimulates the growth of these commensal micro-floras, which stimulate the growth of *Legionella* (Jalila *et al*., 2012; Diederen, 2007).

2.1.3.1 *Legionella* occurrence from Natural Surface Water

Although *Legionella* are ubiquitous in the aqueous environment, few studies examine natural non epidemic surface waters for their presence. Studies clearly demonstrated the widespread occurrence of *Legionella* from natural freshwater sources (e.g, springs, lakes and streams). There are few documented evidence about the occurrence of *Legionella* in groundwater and marine environment as a normal habitat though *Legionella Spp* but had been isolated from estuarine waters (Dimitriadi and Velonakis 2014; Sa´nchez-Buso´ *et al*., 2013; Qin *et al*., 2013; Verı´ssimo *et al*., 2005; Riffard *et al*., 2001). Researches have also revealed that, *Legionella* thrive in biofilms, and interaction with other organisms in biofilms is essential for their survival and proliferation in aquatic environments (USEPA, 1999).

2.1.3.1.1 Natural Water Bodies

Freshwater environments such as lakes, rivers, pond water, hot springs and others are major reservoirs of *Legionella spp* and isolated from watery soils, natural water systems (EPA, 2015; Borges *et al.,* 2012). Even though, the majority of studies focused on the source of *Legionella* contamination in the manmade water systems where they were proliferating than the natural water environment where significant concentration of *Legionella* may present than previously thought. Yet, a good number of scholars have reported the detection of *Legionella* from natural water. For instance, *Legionella* prevalence with a high abundance and diversity reported up to 75% from dam water samples (Chinsembu *et al.,* 2010), 20.8% from a river by (Parthisot, 2010); 8% and 1% from stream and ground water, respectively (Ali *et al*., 2011). Moreover highest richness and highest alpha diversity of *Legionella spp* reported from Canadian watersheds (Peabody *et al.,* 2017). Few reports were obtained from African surface water environment, including studies from South Africa, Nigeria, and Namibia (Ali *et al*., 2011; Chinsembu *et al.,* 2010).

Surveying and monitoring *Legionella* from the aquatic environment has paramount importance to control the possible infection of Legionosis (Ali *et al*., 2011). The *Legionella* concentration in a given sample can be used as a predictive factor for monitoring. Hence, knowledge of the opportunistic pathogens, levels of contamination in various environments will be important to raise awareness, preparedness, prevention and management of future outbreaks of LD (Chinsembu *et al.,* 2010). Besides this, as new *Legionella spp* are identified from time to time from different continents of aquatic environment, there might be room for discovery of new *Legionella Spp* from African water environments as more surveillance of water is conducted.

2.1.3.1.2 Hot Springs /Spa

Hydrothermal or hot springs are unique waters in their mineral content and usually have water temperature above the mean annual air temperature. Hot springs are attractive places known by either for excursion (Olivier *et al*., 2008) or for balneotherapy purposes (Zaini *et al.*, 2013). Hot springs exhibits diverse chemical components and a varied microbial phylogenetic community that may have a potential for biotechnological applications (Chia *et al*., 2015; Memory *et al*., 2015; Akira *et al*., 2003).

Besides the beneficial role to humans, hot springs waters might have physical, chemical and microbial hazards (WHO, 2006). In recent years, *Legionella* bacteria in hot spring water are becoming hazardous emerging public health threats (Jocelyn *et al*., 2017). Outbreaks of LD have been reported from manmade human water systems sources in the built environment including showers, faucets, hot tubs/swimming pools, cooling towers, and fountains (Aaron *et al*., 2017; Atlas, 1999; Doleans *et al*., 2004). *Legionella* can proliferate at temperatures above 25 °C and can be found in high numbers in natural spas using thermal spring water, and they can also grow in poorly maintained hot tubs associated equipment and systems. Hot water distribution systems are potential source of exposure to *Legionella* species (Erica *et al*., 2018; WHO, 2006). Besides the environmental and human factors, recent studies reveled that, hot springs has been associated with opportunistic microbial infection hotspots; and some hot springs shows high genetic polymorphism of *Legionella spp* (Shen *et al*., 2015; Qin *et al*., 2013; Shih, 2010 and Isao *et al*., 2002).

Although both human and environmental factors suitable for *Legionella* existence are available in Africa, most sporadic and community epidemic *Legionella* infections are reported mainly from developed nations. Little or no information is available in Africa about the

prevalence of *Legionella* and the potentiality of being infectious African hot springs in general and Ethiopian hot springs in particular. Ethiopia has numerous hot springs that can be developed for balaneotherapy, recreational and tourism purposes. There is the potential as well as demand for Hot springs in different regions that is why hotels, resorts and lodges investment is growing around hot springs. Furthermost, Ethiopians has been using hot springs mainly for healing of diseases and many of them believe that water from hot springs can relieve from a number of diseases and is considered the cleanest of all (Sisay *et al*., 2015a).Similarly in classical medicine hot springs are used as a cure for diseases Kagamimori *et al*., (2005) and by some, used for religious rites in Egypt and by Jews in the Middle East (Yaowalark *et al*., 2005).

2.1.3.2 Manmade Waters

Many engineered water systems (artificial aquatic habitats) such as cooling towers, water boilers, whirlpools and spas, drinking water distribution networks, shower heads, and dental-unit water lines provide an environment favorable to the growth and multiplication of opportunistic pathogens and are believed to function as amplifiers or disseminators of *Legionella Spp* in potable water (Atlas, 1999; Doleans *et al*., 2004). *Legionella* was isolated from oxidation ponds and Fish ponds (Bercovier *et al.,* 1986) all phases of sewage treatment (Palmer *et al*., 1993); in potable waters and a cooling tower (Gorman *et al*., 1985), in municipal drinking water Systems (States *et al.,* 1987) in hot water of hotels (Borella *et al*., 2005), in ferries and cruise ships (Azara *et al*., 2006), in supermarket mist machine (Barrabeig *et al*., 2010) in hospital decorative water fountain (Palmore *et al*., 2009) in cold water distribution system (Arvand *et al*.,2011).

The growth and survival of microorganisms in manmade water distribution system depend on the interactive effects of factors related to either the structure or management of the network. To mention some factors, temperature, nature of pipe material, available nutrient levels, type and concentration of disinfectants, hydraulic conditions of the system. Among the micronutrients, lower levels of certain metals enhance growth of *L. pneumophila* and especially iron has been linked to *L. pneumophila* extracellular growth, intracellular replication, and virulence. As iron availability in water distribution systems may be linked with the older age and metal constituents of pipes, corrosion products are important factors in the survival and growth of *L. pneumophila* in artificial habitats (Strickhouser, 2007; Masato *et al*., 2013; Solimini *et al*.,2014 and references therein).

L. pneumophila in man-made environmental niches and changes in human behavior have led to legionellosis as a new public health risk that can be associated with serious morbidity and mortality, especially when the infection is not rapidly diagnosed and treated.

2.1.3.2.1 Drinking Water Distribution System

The provision of safe drinking water has been one of humanity's most successful public health interventions. But lack of awareness about potential risks and skills on training of staff and managers on drinking water systems results in outbreaks of waterborne diseases in the community (Hrudey, 2014). According to US Environmental Protection Agency (EPA), there are over 500 waterborne pathogens of potential concerns in drinking water (Ashbolt and Nicholas 2015). *Legionella Spp* are one among other opportunistic water based pathogens found in drinking water distribution system. *Legionella* has the ability to grow and multiply with in the biofilms on pipe wall, sediments and water stagnation and warmer environmental conditions favor them to reach at higher concentration (Ashbolt and Nicholas 2015). As it has

been described somewhere Flemming, (2014) and Percival & Walker, (2002), Biofilms on the wall of mains consists of about 95% of the drinking water micro-biome than in the water. And biofilm in drinking water distribution system is a strong hold or reservoir for different types of microbes including waterborne pathogens like viruses (entero-viruses, Hepatitis A virus, norovirus), protozoa (amoebae, *Cryptosporidium parvum, Giardia spp*.), and bacterial (*Aeromonas spp., Helicobacter pylori, L. pneumophila, Pseudomonas aeruginosa* and *Environmental mycobacteria (EM)* on which some of them are emerging pathogens in recent decades (Mario *et al*., 2005).

Besides being niche for microbes, biofilm aggravates corrosion of iron based water mains, produce bad taste and odor, and decrease residual concentration level of disinfectants Michael *et al.*,(2018), and affects the overall quality of potable water in the system. Testing drinking water quality comprises physical, chemical and microbiological analyses of the water. Though the common indicator organisms used for microbial quality monitoring in drinking water are Total Coliforms (TC) and Fecal Coliforms (FC), currently non- fecal derived pathogens are causing sporadic and epidemic outbreaks.

For such an emerging ,non- fecal derived opportunistic water pathogens, the routine testing of only Total Coliforms and Fecal Coliforms may not be enough to assure microbial quality of given potable water. In the absence of TC and FC, potential opportunistic pathogens like *Mycobacterium spp*., *Legionella spp., Pseudomonas aeruginosa* and *Acanthamoeba spp* might have been detected from water distribution system. Diverse groups of opportunistic waterborne pathogens isolated from sediments of (MDWSTs) municipal drinking water storage tanks USA (Lu *et al*., 2015).

Legionella infection though sporadic and occur in community-dwellers Patrizia *et al.,* (2018) alone accounts for more drinking water-related outbreaks in the United States than all other contaminants combined (Beer *et al*., 2015). Though, most drinking water treatments use Chlorine based disinfectants (residual chloramine) to reduce the abundance of *Legionella spp* Michael *et al.,* (2018), presence of *Legionella* was reported from chlorinated distribution system and the bacterium also has the ability to colonize solid surfaces of water distribution system and formation of biofilm (Anita *et al*., 2012).

Relatively higher level of *Legionella* detection (87%) was reported from the water distribution system of the University hostel of Namibia (Chinsembu *et al*., 2010). Nearly half of the water samples, from public and private water taps across the United States, showed the presence of *L. pneumophila Sg1* in one sampling event, and 16% of taps were positive in more than one sampling event (Maura *et al,* 2014). Likewise, close to half tap water samples were positive for *Legionella* as reported 48% from Flint, USA by (David *et al*., 2016) and 47.5% from Modena, Italy by (Annalisa *et al.,* 2011).

Detection of *Legionella* in samples from water distribution system of buildings also reported varied degree of contamination including 31.5% from Morocco by Mariam *et al.,* (2012), 30.7% from Lazio, Italy retired homes (Patrizia *et al.,* 2018), 29% from Perugia, Italy (Ermanno *et al.,* 2017) and 23.5% from Kuwait residential facilities (Qadreyah *et al.,* 2012) 9.4% from Split, Croatia by (Anita *et al.,* 2012) and 8.5% from Aqaba, Jordan by (Khaled *et al*., 2014). Biofilm development in main water distribution system as source of *Legionella* to tap water premise plumbing system suggested by Michael *et al.,* (2018) and up to 16.9% of biofilm samples in Italy were positive for *Legionella* (Patrizia *et al*.,2018).

To reduce the *Legionella* infection risk, manmade water systems has to be managed properly to control *Legionella spp* at lower concentration in the water system is necessary (Ashbolt and Nicolas, 2015). Water Distribution systems should have to be managed with control strategies with proper disinfectant residual level and minimum level of nutrient availability in the system (Mario *et al.,* 2005). Emphasis has to be given on monitoring opportunistic pathogens in water distribution mains and consumers plumbing system in accordance with the standard or national guidelines (Rakić *et al.,* 2012). Routine monitoring of environmental water for *Legionella* will be important in activities that require reduction of contaminates and for the development of active prevention mechanisms of possible infections (Mariam *et al.,* 2012).

2.1.3.2.2 Hospital Water Distribution System

Water pipes hospitals buildings (premise plumbing) are becoming source of opportunistic microbial infections known as opportunistic premise plumbing pathogens (OPPPs). *L. pneumophila* is the one among others widely known waterborne OPPP (Falkinham *et al.,* 2015) as an emergent concern in owners, managers and occupants of building water systems (Yocavitch *et al.,* 2017).

Water distribution system in health care facilities prone to microbial contamination as the prevalence of free living Amoeba and opportunistic pathogens persist and transmit diseases as a nosocomial infection (WHO, 2002). Contamination of hospital water and *Legionella* infections of hospital patients from hot water system, aerosol generated facilities like showers, faucets, nebulizers and others from health care facilities has been documented (Squier *et al*., 2000).

Although with low incidence, LD is an important cause of community- and hospital-acquired pneumonia (Luigi *et al.,* 2017) but the case fatality rate of healthcare associated LD is quite high, ranging from 38% to 53% (Stout *et al.,* 2011). Hospital-acquired outbreaks of Legionnaires' disease are occurring worldwide and appear to be increasing in frequency (Yocavitch *et al.,* 2017; Yusen *et al*., 2011). Despite progress in public health and hospital care, infections continue to develop in hospitalized patients, and may also affect hospital staff (WHO, 2002).

The incidence of Legionnaires' disease appears to be increasing, both community-acquired and hospital acquired level (Yocavitch *et al.,* 2017; Yusen *et al.,* 2011). The manmade environmental niches and changes in human behavior have led to *Legionella* infection as a new public health risk that can be associated with serious morbidity and mortality, especially when the infection is not rapidly diagnosed and treated (Mariam *et al*., 2013 ; Julianne *et al.,* 2015; Muchesaa *et al,* 2018; Bartley *et al*., 2017).

It has been documented that, among the tested hot and cold water samples from hospital water distribution system, 74.77% from eastern Poland Sikora *et al*., (2015), 67.1% from Tuscany-Italy by Antonella *et al*., (2011), 66% from Iran by Asghari *et al*, (2013) , 35% from Hesse, Germany by Arvand *et al*.,(2011), 33.33% of the sample from Messina hospital ,Italy by Laganà *et al.,(*2014), 33% from south western Greece by Fragoue *et al.*, 2011), 23% Iran by Asghari *et al,* (2013) , 23.7% in Italy (Alessandro *et al*., 2015), 11.6% from Gabon by Ehrhard *et al*.,(2015) were positive for *Legionella spp*.

Besides this, the prevalence of amoeba-associated *Legionella pneumophila* from hospital water systems are the driving force for the survival, growth and evolution of the pathogenicity linked to nosocomial infection by *Legionella* (Muchesa *et al*., 2015; Mariam *et al*., 2013).

Hence the presence of free-living amoeba (FLA) from the hospital water system was detection confirmed as high as 88.7% (Muchesa *et al.,* 2015) which is an additional water quality problem. With this and other comorbidities, hospitalized patients might have an increased risk to become infected with *Legionella spp* (Ehrhard *et al*., 2015).

Efficient LD management requires rapid diagnosis, treatment, epidemiological awareness and ecological studies on water distribution system on *Legionella spp* are essential to better understand their sources, mechanism and enabling factors of *Legionella* entry into man-made water systems (Mariam *et al.,*2013; Jeffrey *et al.,* 2015). Even though , additional information is needed for the optimal prevention and control measures, in a healthcare facility , a proactive approach of periodic testing or culturing for *Legionella Spp* along with proper water treatment even in the absence of cases is the best approach to avoiding large-scale disease outbreaks (Yocavitch *et al.,* 2017 ;Yusen *et al.,* 2011). Supplemental disinfection (On site disinfection) of the hospital water distribution system is a good prevention approach to *Legionella* infection (Lin *et al.,* 2011; Stout *et al*., 2011; Sikora *et al*., 2015; Antonella *et al.,* 2011).

Albeit hospital water safety is a constant challenge for healthcare epidemiologists, safety officers, engineers, and administrators (Brooke and Tara, 2014), the regular monitoring surveillance of microbial condition of water distribution systems in hospitals and other health care facilities is an important and necessary element of the control of infections caused by *Legionella* and other opportunistic organisms (Sikora *et al*., 2015; Fragoua *et al.,* 2012; Antonella *et al.,* 2011). Health care facilities should have prospective water safety plans that include preventive measures, which is preferable than remediation of contaminated hospital water distribution system (Brooke & Tara, 2014).

2.1.3.3 *Legionella* occurrence in Soil

Legionella spp had been isolated from mud and sandy, moist soil on the edge of streams containing the bacteria (Tatiana *et al.,* 2012). Same document noted a lack of data indicating whether or not soil is involved in the transmission of *Legionella* to humans although excavations and other soil disturbances had been associated with some *Legionella* epidemics. At that time, *Legionella* had only been found from mud or moist soil. *L. longbeachae* isolated from three samples of potting soil were able to persist for seven months in two potting mixes stored at room temperature. The researchers concluded that the isolation and prolonged survival of *L. longbeachae* in potting mixes suggest that soil rather than water is the natural habitat of this species and may be a source of human exposure.

2.1.3.4 *Legionella* occurrence in Air

Legionella can be found in air as part of aerosols showing that air is an important component for *Legionella* transmission from the aquatic environment to the human respiratory system. Hence, aerosol-generating water systems that had been linked to disease transmission include cooling towers, evaporative condensers, plumbing equipment (faucets, showerheads, and hot water tanks), humidifiers, respiratory-therapy equipment (nebulizers), and whirlpool baths. In most cases, disease outbreaks resulting from *Legionella* aerosolization have involved indoor exposure and outdoor exposure to within 200 meters (USEPA, 1999).

As it has been outlined by Mercante & Winchell (2015), *Legionella* from freshwater sources is distributed at low concentrations from points of water purification but can colonize downstream local plumbing networks and amplifies under permissive environmental

conditions. Subsequent aerosolization of water from the system exposes a human population, which may include individuals with increased susceptibility leading to a potential disease spectrum. More susceptible individuals (due to age or underlying medical conditions) are at a higher risk of LD than those less susceptible, and both groups are at risk for Pontiac fever. The route of LD caused by contaminated soil is less well understood but also appears to involve aerosol exposure. The overall route of *Legionella* dissemination from natural waters to development of LD is adopted from Mercante and Winchell, (2015) and presented in Figure 1.

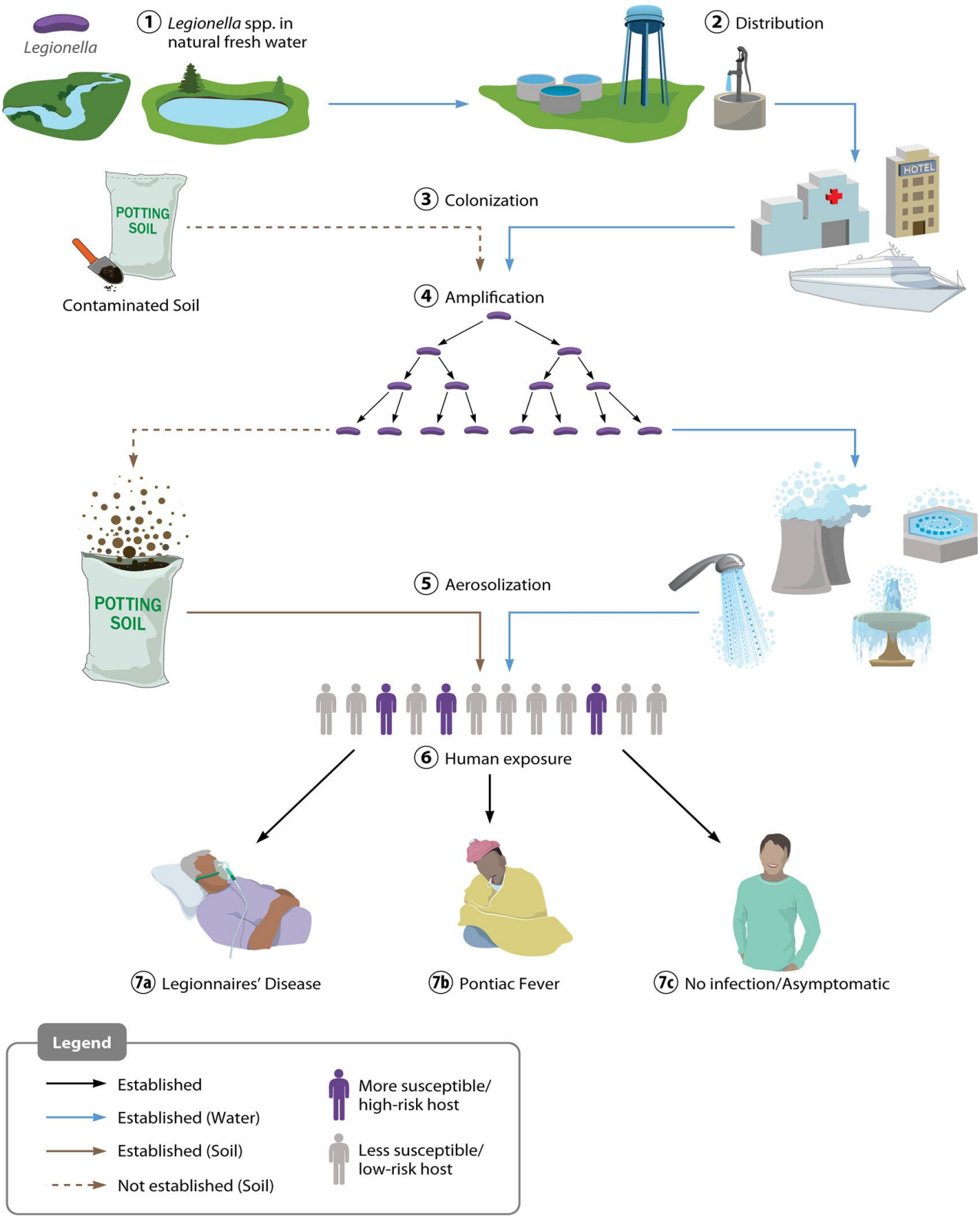


Figure 1 Route of *Legionella* dissemination from natural waters to development of LD

Courtesy: Mercante and Winchell, (2015)

2.1.4 Epidemiology

Epidemiological studies indicate that *Legionella* is an opportunistic pathogen. The case-mortality rate of adequately treated Legionnaires' disease varies from 7% to 24% Steinert *et al.,* (2002) with elderly and immuno-compromised patients being most susceptible but in some conditions the fatality my rage from 5% to 80% (WHO, 2007). According to Steinert *et al*., (2002) clear determination of infectious dose of *Legionella* is difficult due to existed differences in host susceptibility and bacterial virulence. The incidence of Legionnaires' disease depends on several factors, including the extent of contamination of the water reservoir by the organism, susceptibility of the population exposed to that water, and the degree or intensity of the exposure of the patient to the water reservoir (Vergis *et al.,* 2000). Furthermore, available skills and efficiency of laboratory tests are also important factors for diagnosis. The aerosol generating devices from potable water systems of hospitals, hotels, office buildings, work places, apartment buildings are common sources of Legionnaires' disease (Guyard *et al*., 2013; Steinert *et al*., 2002).

While legionellosis is widely distributed geographically throughout the world, most cases have been reported from the industrialized countries. The true incidence of legionellosis is difficult to determine because identification of cases requires adequate surveillance and research suggests that LD is under reported to national surveillance systems. Mainly LD recognition depends on the physician awareness of the disease and resources available to diagnose it.

2.2 *Legionella* Infection Prevalence and Distribution:

The investigation of a number of epidemic and sporadic cases has shown that *L. pneumophila* is a common cause of both community-acquired and nosocomial pneumonia. Primarily the immune-compromised and elderly are highly susceptible for *Legionella* infections (Steiner *et al.,* 2002). Nevertheless in certain cases reported legionellosis cases have increased among middle-aged adults substantially in recent years, particularly in the Eastern United States. Cigarette smoking, advanced age, chronic lung disease, immunosuppression including receipt of corticosteroids, organ transplant recipients and others are well-established risk factors for Legionnaires' disease (Vergis *et al*., 2000 and USEPA, 2015).

Cases of LD may be sporadic or occur as part of an outbreak. Sporadic cases are reported throughout the year, but most cases of epidemic infection occur in the summer and autumn, presumably because warmer weather encourages proliferation of the bacteria in water (Diederen, 2008). Albeit, limited studies have been done regarding socioeconomic and occupational risk factors for community-acquired cases; estimated 8,000–18,000 persons are hospitalized for Legionellosis each year in the United States; about 5% - 30% of case-patients die (AWT, 2019).

For example, the cases of LD in the USA have tripled in the past decade from 1110 in 2000 to 3522 in 2009 with increased incidence rate from 0.39 to 1.15 per 100 000 people (CDC, 2011). Similarly LD increased 279%, from 1,110 in 2000 to 4,202 in 2011 showing an increased incidence rate of 249% from 0.39 per 100,000 persons in 2000 to 1.36 per 100,000 within the same period (CDC, 2011). According to Lu *et al*. ,(2014) , *Legionella pneumophila*, accounts for some 30% of U.S. drinking water-related outbreaks and about 80%

of drinking water-related deaths in the United States . The incidence rate of LD in Europe is 3-8% and the Pontiac fever is around 80-95% (Kozioł *et al*., 2014 and Farnham *et al*., 2014).

2.3 Trend Analysis of Legionellosis

Data from developed nation shows that the trend of Legionellosis is increasing because of the increased level of awareness about the pathogen and advanced detection methods. For example, the cases of LD in the USA have tripled in the past decade from 1110 in 2000 to 3522 in 2009 and the incidence rate increased from 0.39 to 1.15 per 100 000 people during that time. LD is on the rise in USA as presented by AWT (2019), in Figure 2, and in some parts the incidence of LD followed a socioeconomic gradient, with highest incidence occurring in the highest poverty areas. Among patients with community acquired cases, the probability of working in transportation, repair, protective services, cleaning, or construction was significantly higher for those with LD than for the general working population (Farnham *et al.*, 2014). Besides the rise in the number of seniors and other people at high risk for infection in the country, amplification of *Legionella* concentrations within building plumbing systems is a major contributor to increased risk, and distribution systems represent the source of the original inocula (WHO, 2014 and Vergis *et al*., 2000).

Legionella spp., are frequently identified in drinking water biofilms following water treatment. One species, *Legionella pneumophila*, accounts for some 30% of U.S. drinking water-related outbreaks and about 80% of drinking water-related deaths in the United States and about estimated 25,000–100,000 LD cases annually (Lu *et al*., 2014 and Riffard *et al*., 2001).

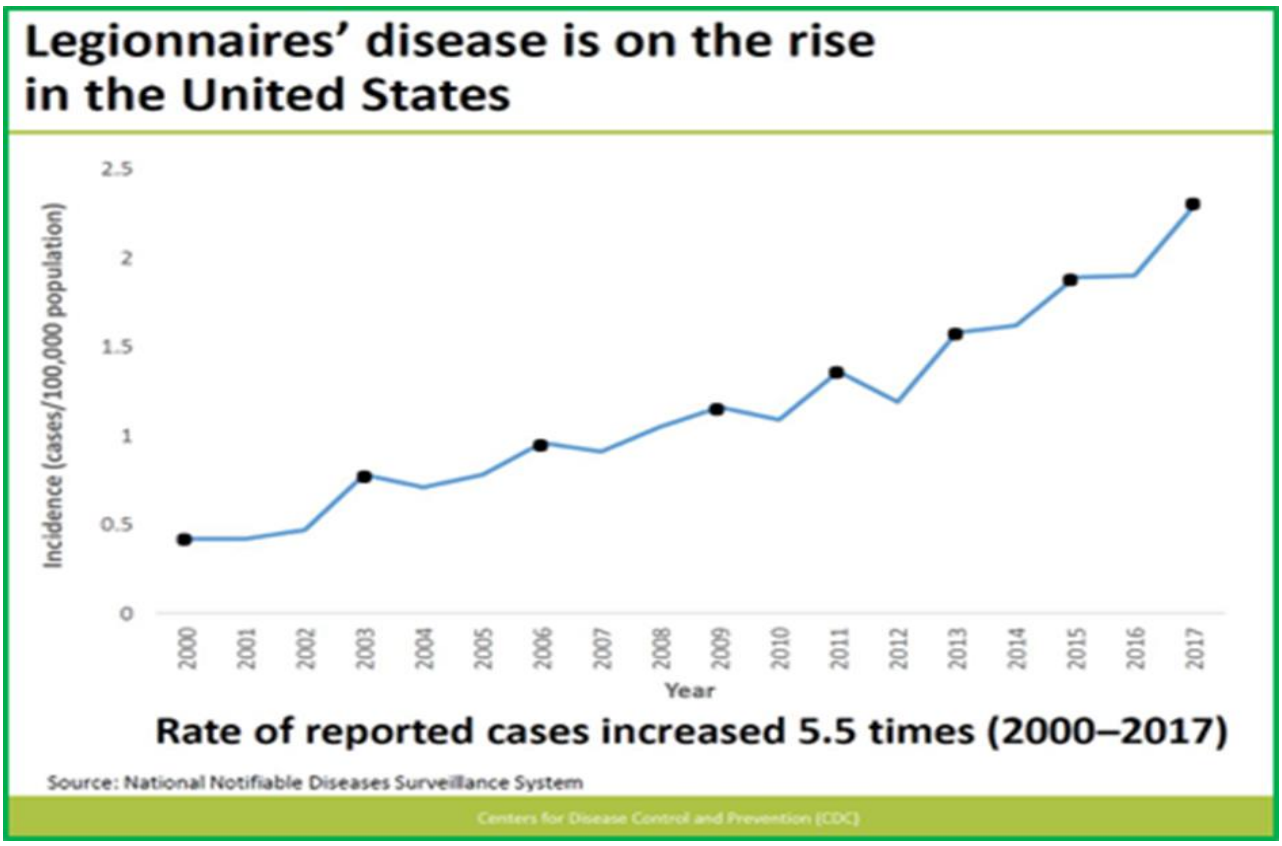


Figure 2 Annual rate of reported LD cases in USA from 2000-2017

Courtesy: AWT, 2019: CDC report 2018

WHO (2007), reported an increasing trend of *Legionella* incidence in (EU) European Union. The report indicated the diversity of exposure to *Legionella* infection from different environmental sources as nosocomial, travel associated, and community acquired and some not known was observed. Similarly WHO (2007), ECDC (European Centre for Disease Prevention and Control) reported an increasing trend of causes of LD incidence as presented in Figure 3 and 4.

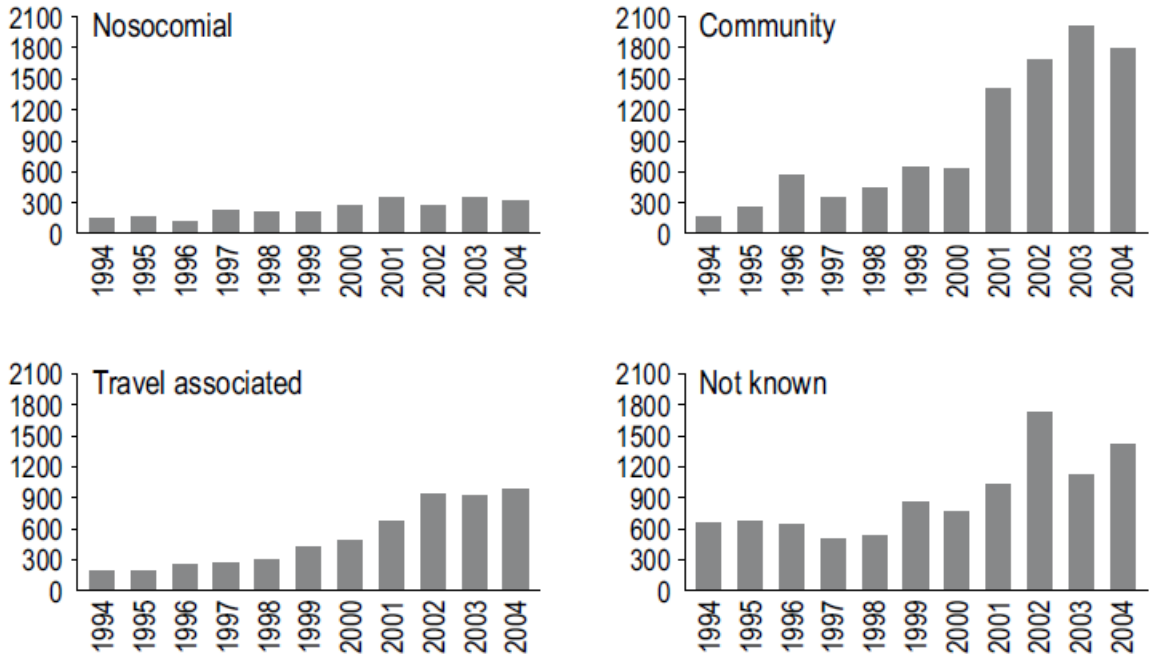


Figure 3 Annual reported LD cases and LD by category exposure in EU (1994–2004)

Courtesy: WHO, 2007

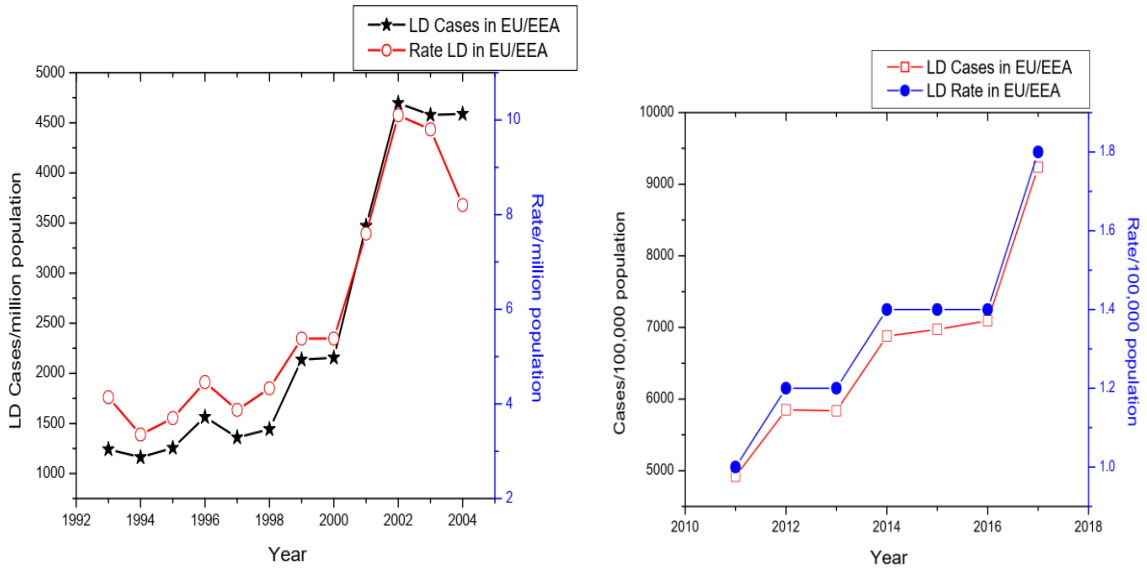


Figure 4 Notification rates of LD in the EU/EEA (1992–2018)

Courtesy: ECDC, (2009-18)

LD incidence reported from fast growing developing countries like China (Hong Kong) and others is coming in recent years. As it has been observed from a five year incidence data in Figure 5, comparable incremental trend of LD incidences have been observed (Wong, 2018).

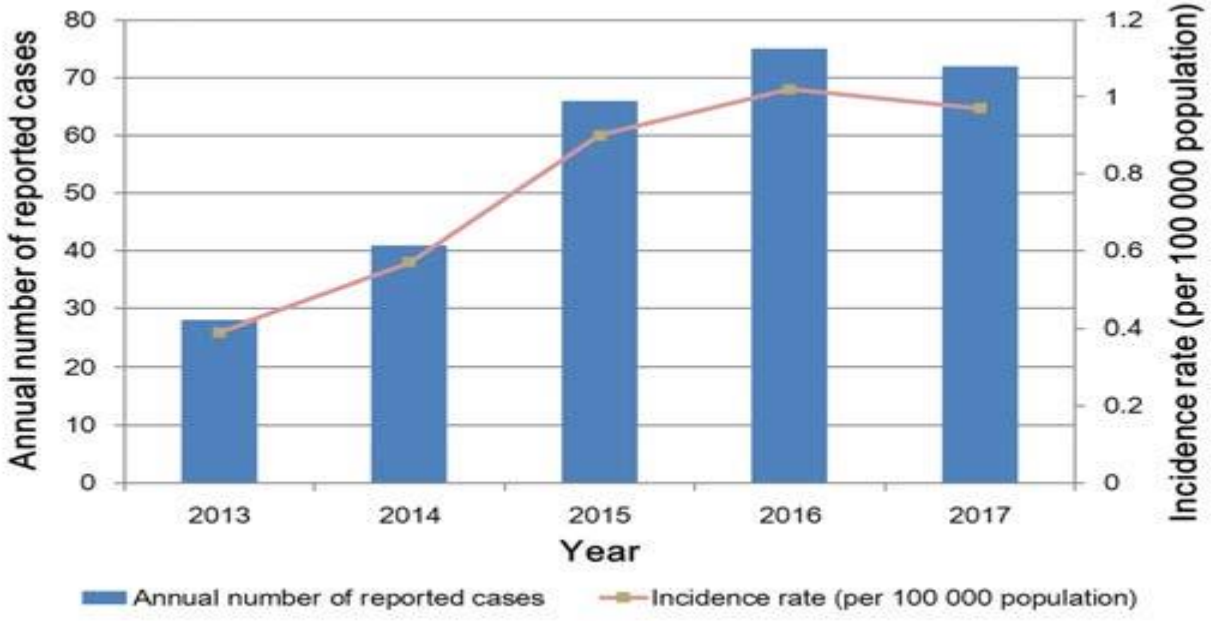


Figure 5 Annual number and incidence rate of reported LD cases in Hong Kong, 2013-2017

Source: Wong, (2018)

Commonly, a rising trend in the number of reports of LD has been observed since 1990. There were several factors which contributed to this increase. Firstly, the continued increase in case ascertainment of Legionellosis was partly attributable to publicity awareness about the disease and the increasing use of the urinary antigen test (UAT) and molecular methods od diagnosis in developed nations Janet *et al*., (2002) however, LD related information's and data are not available in most developing countries.

2.4 Distribution of Legionellosis by Age and Gender

Marked association between age, gender, and human legionellosis reported. Compare to the gender, LD notifications has been more in Males and the elderly (older than 50 years) than the Females counterparts with the same age level. It has been reported that that *Legionella* notification rate for males increased gradually over the adult years while the notification rate for females started to increase for those aged over 50 years, however the magnitude was much smaller than that observed in males (Farnham *et al.,* 2014). Males are at greater risk of LD infection, partly because males are more likely to be heavier smokers and therefore may tend to have inferior respiratory and general health. Also, advancing age leads to the deterioration of general health, which makes the elderly more vulnerable to the disease when exposed to *Legionella* (Janet *et al*., 2002).

Despite the fact that LD in developed nations is increasing, still under-diagnosis and underreporting of *Legionella* are thought to lead to a significant under-estimation of incidence of LD in many countries. This is because, the incidence and the reported values of LD depends up on several factors like the extent of the water contamination level of the water susceptibility of the population, degree of intensity of the exposure, experience of testing lab and the availability of specialized tests (HPSC, 2009). In line with this, pneumonia is mostly treated with antibiotics that can cover *Legionella* and patients recover without the need to establish the cause of pneumonia not being notified (CDC, 2011 and Vergis *et al*., 2000). Though, there are available predisposing factors in developing countries enough information is not available with respect to *Legionella* prevalence and infection.

2.5 Transmission and Pathogenesis

2.5.1 The Transmission Cycle

The transmission of *Legionella* infection is not through fecal pollution rather the transmission is associated with the inhaling or aspirating aerosolized of *Legionella* contaminated droplets (1-5 μm diameter) or mist (USEPA, 2015). *Legionella* infections have frequently been associated with sources at distances of up to 3.2 kilometers and in some evidences infection may be possible at even longer distances (Mekkour *et al*., 2013). Additionally, aerosol formation, aspiration and direct installation of bacteria in the lungs in connection with manipulation of the respiratory tract are the most common means of *Legionella* infection in hospitals environment. In patients with chronic obstructive pulmonary disease (COPD), the protective function of the airways is impaired and the disease is associated with aspiration. *Legionella* can be introduced into the airways by means of contaminated puritan nebulizers. Besides this, the virulence is vital factor in the survival of *Legionella* in aerosols, with the most virulent strains surviving longer than their less virulent counterparts (Jalila *et al*., 2012).

The exact dose of *Lp* required to infect humans is not definitively known and in some researches ingesting *Lp* has not been shown to cause illness. The dose is most probably influenced by host susceptibility (AWT, 2003). But potential health risk associated with the cell densities increases above 10^4 to 10^5 CFU/Liter of water (Mekkour *et al*., 2013; Al-Matawah *et al*., 2012 and WHO, 2007).

Legionnaires' disease is always transmitted from the environment to humans, and thus constitutes an environmental disease that cannot be transmitted from person to person. Despite years of accumulated clinical experience and intense epidemiological research, a satisfactory

explanation for the lack of person-to-person transmission of Legionnaires' disease does not exist (Jalila *et al*., 2012; Hoffman *et al*., 2008 and WHO, 2007).

2.5.2 Clinical Manifestations

Legionellosis classically presents two distinct clinical entities, pneumonia with severe multisystem disease (LD), and Pontiac fever, a self-limited flu-like illness(WHO, 2007). It is estimated that Legionellosis affects 25 000-100 000 persons annually in the United States. In a series of studies from North America and Western Europe, 1-13% of all pneumonia cases were associated with this pathogen. Unless specifically targeted, distinguishing LD from other forms of pneumonia is difficult and hence, many cases of Legionellosis go probably unreported (AWT, 2003). It is not possible to distinguish clinically patients with LD from *Pneumococcal pneumonia* patients. Several prospective studies have shown that the two diseases have nearly identical clinical and radiological findings, and non-specific laboratory test results may not differentiate between the two diseases (Mekkour *et al*., 2013; Diederen, 2008; Fields *et al*., 2002; Vergis *et al*., 2000).

As well, many persons who are infected with *Legionella,* as proven by seroconversion, remain asymptomatic. But the symptomatic patients can show some common clinical manifestations. The clinical symptoms, incubation period, duration, case fatality and attack rate of *Legionella* developed by WHO (2007), are presented in Table 1.

Table 1 Main characteristics of Legionnaires' disease and Pontiac fever

Characteristic	**Legionnaires' disease**	**Pontiac fever**
Incubation	Period 2–10 days, rarely up to 20 days	5 hrs–3 days (most commonly 24–48 hrs)
Duration	Weeks	2–5 days
Case fatality rate	Variable depending on susceptibility; in hospital patients, can reach 40–80%	No deaths
Attack rate	0.1–5% of the general population 0.4–14% in hospitals	Up to 95%
Symptoms	Often non-specific • Loss of strength (asthenia), • High fever, Headache, Nonproductive, dry cough • Sometimes expectoration blood-streaked Chills, Muscle pain, • Difficulty in breathing, chest pain, • Diarrhea (25–50% of cases), • Vomiting, nausea (10–30% of cases) Central nervous system manifestations, such as ✓ confusion and delirium (50% of cases), ✓ Renal failure, ✓ Hyponatraemia (serum sodium <131 mmol/litre), ✓ Lactate dehydrogenase levels >700 units/ml, ✓ Failure to respond to beta-lactam antibiotics or aminoglycosides, ✓ Gram stain of respiratory specimens with numerous ,neutrophils and no visible organisms	Influenza-like illness (moderate to severe influenza) ❖ Loss of strength (asthenia), tiredness, ❖ High fever and chills ❖ Muscle pain (myalgia), Headache ❖ Joint pain (arthralgia), ❖ Diarrhoea ❖ Nausea, vomiting (in a small proportion of people) ❖ Difficult breathing (dyspnoea) and dry cough

Source: WHO, 2007

2.5.3 Community-Acquired Pneumonia (CAP)

The term "community-acquired pneumonia" (CAP) refers to infections from industrial sites, shopping centers, restaurants, clubs, leisure centers, sports clubs, and private residences are categorized under community acquired *Legionella* infection. CAPs have a high rate of hospital admission, with less than 1% being managed at home and LD can account for up to 30% of CAPs requiring admission to intensive care (WHO, 2007). Appropriate empirical treatment depends on knowledge of the pathogens commonly responsible. However, assessing the etiological significance of identified organisms is often difficult, particularly with sputum

isolates that might represent contamination with oropharyngeal flora (Farnham *et al*., 2014; Fiumefreddo *et al*., 2009 and WHO, 2007).

2.5.4 Hospital-Acquired Pneumonia (HAP)

Hospital-acquired pneumonia (HAP) also called nosocomial infection, is considered the second most frequent cause of nosocomial infection, accounting for 15 to 30% of these infections (Jalila *et al*., 2012). Hospitals represent ideal locations for Legionnaires' disease transmission: at-risk individuals (immunocompromised patients) are present in large numbers; plumbing systems are frequently old and complex (Fields *et al.,* 2002; WHO, 2007). Legionellosis, in hospitals (health care facilities) usually is acquired by inhalation or aspiration of *Legionella* from contaminated medical equipment and environmental sources (Jalila *et al.,* 2012).

Among cases of *Legionella pneumonia* from 1980 to 1998, the percentage of cases identified as hospital acquired ranged from 25% to 45%. Mortality associated with hospital-acquired *Legionella pneumonia* (28%) is approximately double the mortality for community-acquired cases (14%). Nosocomial LD is vastly under diagnosed primarily because cultures on multiple selective media are generally not available in house (WHO, 2007). According to Jalila *et al*., (2012) and WHO, (2007), contaminated potable water supply is an important source of both nosocomial and community acquired *Legionella* infections.

The presence of *Legionella* species in the hospital water supply suggests that patients in the hospital may be at risk for hospital-acquired *Legionella pneumonia* and triggers the routine implementation of *Legionella* diagnostic tests for patients with hospital-acquired pneumonia (Stout *et al.,* 2007; WHO, 2007; Vergis *et al*., 2000).

2.5.5 Travel Associated Legionella (TALD)

A travel-associated Legionnaires' disease (TALD) is an infection observed after inhaling contaminated aerosols from manmade water facilities, hotels, cruise ships, camp sites, shopping centers, restaurants, clubs, leisure centers, and sports clubs. Report from developed countries revealed that the prevalence of travel associated diseases is increasing from time to time. For instance in Europe, a total of 864 cases of travel-associated LD was reported with onset of disease in 2010. This is an increase (+5.6%) compared with the 818 cases reported in 2009 but does not reach the peak of 947 cases observed in 2007. The 864 reported cases had made 1,279 visits to accommodation sites around the world and they visited a total of 66 countries in the 2–10 days before onset of disease. Legionnaires' disease is still under ascertained in most European countries since specific testing for *Legionella* in patients with pneumonia is not a routine procedure (ECDC, 2010).

Moreover, in 2012, 831 cases of TALD were reported by 20 EU/EEA countries, Croatia and the United States of America. This was 8% higher than the 763 cases reported in 2011. Five countries (France, Italy, the Netherlands, Spain, and the UK) reported 77% of all TALD cases of an increasing trend of TALD in EU reported by DeJong *et al*., (2013) as shown in Figure 6. For 2016, 1082 TALD cases were reported through near-real-time surveillance: which was 5% fewer than in 2015, but 14% and 37% more than in 2014 and 2013 respectively (ECDC,2016). Cases of Legionellosis in hotels have often received extensive and damaging publicity, with significant economic impacts due to reduced patronage (WHO, 2011).

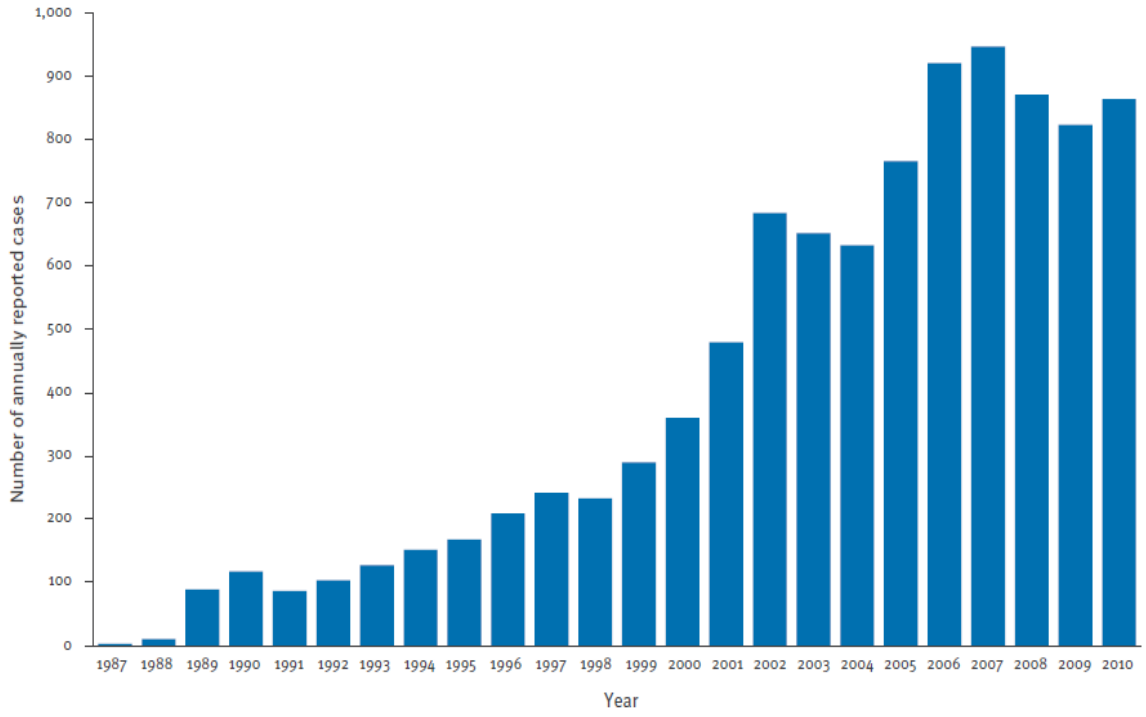


Figure 6 Annual reported cases of TALD from 1987-2010 in EU/EEA countries

Source: De Jong *et al*., (2013)

Numerous reports have demonstrated that, the major sources for Legionnaires' disease are the potable water systems of large buildings including hospitals, nursing homes, and hotels (Vergis *et al*., 2000). The general risk factors for LD in manmade environments includes water in the distribution system which is not used continuously; water distribution system which has "dead legs" (dead branches) of the installations and the intake of water; medical equipment's like dialyzers, respirators, spirometery, inhalers, dental turbines; equipment's of hydrotherapy; air conditioning systems; cooling towers in industrial plants (Kozioł *et al.*, 2014).

2.5.6 Pathogenesis

Currently, about 20 *Legionella species* have been documented as human pathogens on the basis of their isolation from clinical material (Mekkour *et al.,* 2013; Diederen, 2008; Steinert *et al.,* 2002). *L. pneumophila* is the most frequently implicated species in pneumonia, accounting for about 90% of infections, while *L. micdadei* (Pittsburgh pneumonia agent), *L. bozemanii, L. dumoffii, L. longbeachae,* and other species together account for the remaining 10% (WHO, 2007; Fields *et al.,* 2002; UEPA, 1999).

Strains of *L. pneumophila* differ in virulence. Multiple *Legionella* strains may colonize the water distribution system, but only a few strains are responsible to cause disease in patients exposed to that water. There are about 15 serogropus of *L. pnuemophilia* but serogroups 1, 4, and 6 are responsible for the majority of human infections (WHO, 2007). Even though, the virulence mechanisms of *L. pneumophila* are complex and not fully understood, the primary feature of the pathogenesis of *Legionella* is their ability to intra-cellular multiplication (Fields *et al.,* 2002; WHO, 2007).

The mechanisms of *Legionella* to infect mammalian and protozoan cells are related, using common genes and gene products. The ability of *L. pneumophila* to parasitize human macrophage and to cause human disease is thought to be a consequence of its prior adaptation to intercellular growth with in various protozoan hosts (Steinert *et al.,* 2002). This is most likely due to bacterial acquisition of eukaryotic genes during its coevolution with amoebae and adaptation to intercellular life with in primitive eukaryotic hosts. *Legionella* have a similar life cycle within protozoa and human macrophages; however, there are differences in the mechanisms used to enter and exit from the respective host cell types (Barry *et al*., 2002).

The interaction of virulent *Legionella* with phagocytic cells can be divided into several steps: first it starts with the binding of microorganisms to receptors on the surface of eukaryotic cells; then penetration of microorganisms into phagocytes follows; involve an escape mechanism from bactericidal attack; formation of a replicative vacuole (a compartment within the cell where bacterial replication occurs); intracellular multiplication and killing of the host cell (Steinert *et al.*, 2002). Specifically, once *Legionella* enters the lung of an infected person both virulent and non-virulent strains are phagocytosed by alveolar macrophages and remain intact inside the phagocytes.

However, only virulent strains can multiply inside the phagocytes and inhibit the fusion of phagosomes with lysosomes. During phagocytosis, *Legionella* initiate a complex cascade of activities, including: inhibition of the oxidative burst; reduction in phagosome acidification; blocking of phagosome maturation; and changes in organelle trafficking, *Legionella* thus prevent bactericidal activity of the phagocyte, and transform the phagosome into a niche for their replication (Giulia *et al*., 2018 , Yousef *et al.*, 2013).

The organisms can leave the host cell after temporal pore formation- mediated lysis or can remain within an encysted amoeba. Together with the flagellum and the pili, certain bacterial surface proteins are involved in the adherence and entry of *Legionella* into alveolar macrophages and protozoa (Steinert *et al.*, 2002). These proteins include: the major outer membrane protein (MOMP); the heat shock protein (Hsp60); the major infectivity potentiator protein (MiP). MOMP binds the complement component C3, and mediates the uptake of *L. pneumophila* via macrophage receptors for the complement components CR1 and CR3 Phagocytosis of *L. pneumophila* also occurs by a complement-independent mechanism. The organism is phagosytosed through a process mediated by monocyte complement receptors,

complement component C3 and the major outer membrane protein and the macrophage infectivity potentioator protein (MiP) on the surface of *L. pnemophilia* (Yousef *et al*., 2013).

L. pnemophilia evades intracellular distraction by inhabiting phagosome lysosome fusion. Gens responsible for this evasion have been identified as dot (defective organelle trafficking) and icm (interacellular multiplication) gen. *L. pnemophilia* forms pores in the lipid membranes of a number of cell types which allows for entrance of small molecular weight substances to enter, thus preventing the acidification of the phagosome which proceeds phagosome lysosome fusion. Virulence factors apart from dot/icm gens vary from serotypes to serotype (Giulia *et al.*, 2018; Yousef *et al*., 2013; Barry *et al*., 2002 ;WHO, 2007).

The ability of beta lactomase production; exotoxin production including hemolysis, cytoxin, deoxyribonuclease, ribonuclease, and other protease; ability to produce a weak lipo polysaccharide endotoxin,which can activate the classical complement pathway; and flagella with surface antigens that are recognized by monoclonal antigen 2 are important virulent properties of *L. pneumophila*. When inhaled into the lung, *L. pneumophila* can cause acute alveolitis and bronchiolitis (Yousef *et al.,* 2013). In patients with Legionnaires' disease, the alveolar exudate typically consists of equal numbers of polymorphonuclear cells and macrophages, with fibrin, red blood cells, proteinaceous material, and significant amounts of cellular debris (Yousef *et al*., 2013; Barry *et al*., 2002; WHO, 2007; Giulia,*et al*., 2018).

Legionella colonize and persist in a wide range of extracellular and intra-organismic environmental niches, including biofilms, protozoa and nematodes (Hilbi, *et al.,* 2011). Specifically, *Legionella* can multiply intra-cellular in some protozoa members like amoebae (e.g. *Acanthamoeba, Hartmanella, Naegleria* and etc) and ciliates (*Tetrahymena spp)* as facultative intracellular parasites (Hilbi., *et al.,* 2011; AWT,2003; Fields *et al.,* 2002 and

Steinert *et al*., 2002) . The Schematic overview of the *Legionella* growth cycle is presented in Figure 7.

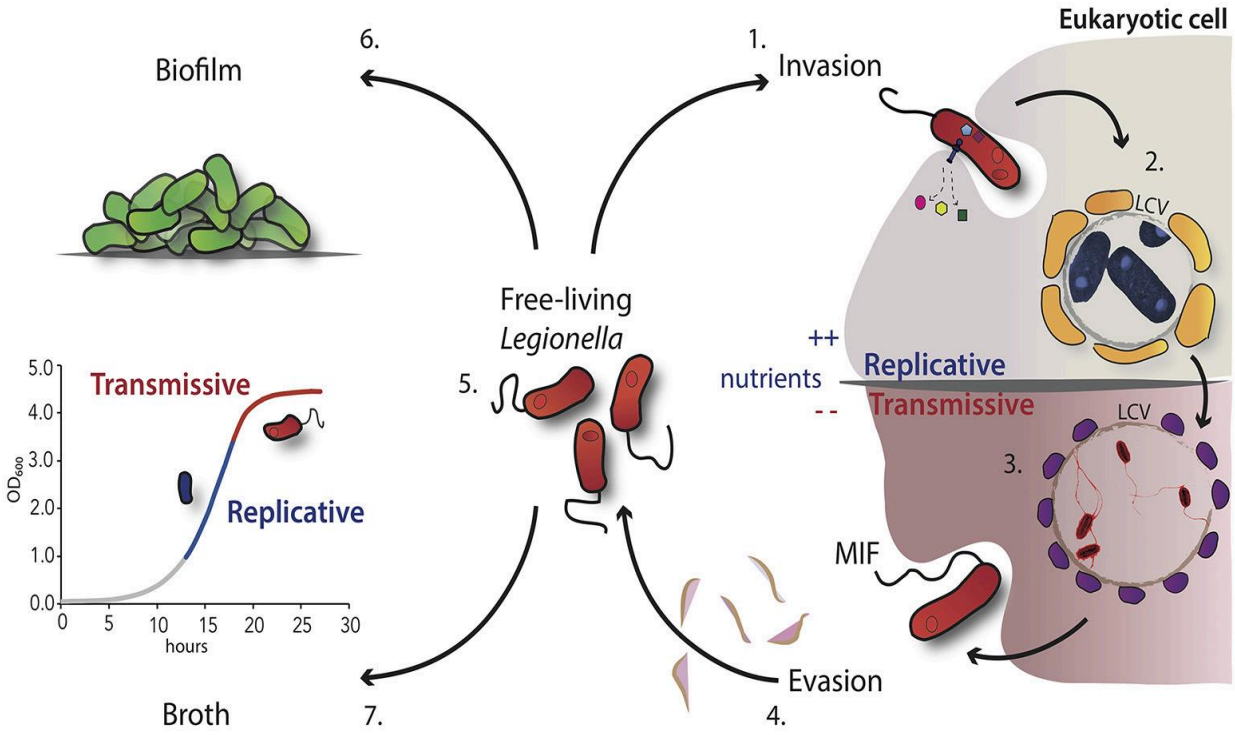


Figure 7 Schematic overview of *Legionella* growth cycle

Source Giulia *et al.*, (2018)

Notes:

1. *Uptake of virulent L. pneumophila by the host cell like protozoa or macrophages through convention or coiling phagocytosis. 2. After internalization, the bacteria evade the phagosome-lysosome fusion and start the intracellular multiplication within the LCV, which is surrounded by vesicles (in yellow) rich in lipids and proteins. 3. Nutrient starvation induces the activation of the stringent response and morphological changes. Bacteria express the transmissive traits such as motility (flagella) and become cytotoxic. 4 These infectious bacteria are able to lyse the vacuolar membrane and are released in the extracellular environment. 5.Free-living transmissive bacteria may start a new cycle or persist in the extracellular environment as planktonic form. 6. Alternatively, L. pneumophila may be associated within biofilms, either in natural fresh-water habitats or artificial ones. 6. In broth culture, L. pneumophila displays also a biphasic life cycle, which closely mimics the replicative and transmissive intracellular forms,*

Generally *Legionell*a infection and growth in protozoa members is an important mechanism of survival and protection from disinfectants and other adverse environmental conditions (USEPA 1999). The broad protozoa host spectrum in the environment and the exploitation of very basic cellular mechanisms of eukaryotes obviously allow *Legionella* to infect human cells. Consequently, it has been suggested that, protozoa are the driving force in the evolution of the pathogenicity of *Legionella.* Amoebae are the primary natural hosts of *L. pneumophila* and constitute the central axis around which the life cycle of *L. pneumophila* revolves (Mekkour *et al*., 2013; Hilbi *et al*., 2010; Hoffman *et al*., 2008; Steiner *et al*., 2002).

2.5.6.1 Pathogenesis Factors

Though the patient populations, the mode of transmission and potential environmental reservoirs are key issues for the likely occurrence of LD, implicating both host (human) and environmental factors to play significant roles as discussed below.

2.5.6.2 Human Factor

LD is an opportunistic infection seeking to get certain human comorbidity factors that reduce or weaken the immune status of an individual. Factors comprise advanced age (getting old), being an alcoholic, smokers, patients of non-communicable diseases (like diabetes, chronic obstructive pulmonary disease and cancer lung cancer or leukemia), and taking either corticosteroid or other forms of immunosuppressive therapy are susceptible groups of human population for *Legionella* infection. Moreover, having recent surgery, transplant recipients, immunosuppressed children and AIDS patients may also be at increased risk. The mentioned

human factors are responsible to reducing the immune status of an individual exposing to opportunistic infection including *Legionella* and others (Jalila *et al*., 2012).

2.6.5.3 Environmental Factors

The symbiotic relationships that *Legionella* has with other microorganisms helps them to survive in wide range of natural and manmade environmental set ups (USEPA, 1999). The environmental factors contributing to the colonization of artificial water distribution systems comprises; increased temperature (optimal between 20°C to 50°C), the presence of sludge, mud, corrosion products, biofilm, and biological agents (other bacteria, protozoa), the stagnation of water installation (no recirculation, "dead legs" of the installation), and too low concentration of the disinfectant(WHO,2007; AWT, 2003).

The other important conditions that may lead to the occurrence of microbes and the formation of biofilms in water distribution systems, include source water with high dissolved or particulate organic matter poor water temperature control; changes in water flow and stagnation; neutral pH in drinking-water; microbial interactions with pathogens such as *Acanthamoeba, Hartmanella* and *Naegleria*; low oxygen; piped distribution system and certain pipe materials; inadequate cleaning and maintenance of distribution systems; loss of disinfectant residual; water main failures and breaks; and conditions of storage facilities, such as high volume tanks that support stagnation and stratification or uncovered storage facilities (WHO, 2014).

2.5.6.3.1 Influence of Temperature

Naturally occurring *L. pneumophila* survives and multiplies in water at temperatures between 20 ºC and 50 ºC, (optimal tem. range of 35–45 ºC) (AWT,2003). But *Legionella* has also been isolated from hot-water systems up to 66 ºC; temperatures above 70 ºC destroy almost instantly (WHO, 2007). But *Legionella* exhibiting survival in natural warm waters of to 60°C and artificially heated waters of 66.3°C(USEPA,1999) Some research findings outlined that growth of all strains of *Legionella* tested decreased at temperatures above 44–45 ºC, with the growth-limiting temperature being between 48.4 ºC and 50.0 ºC (Ohno *et al.,* 2003)

Complex water systems, such as warm-water plumbing systems, air conditioners and hot tubs (also known as spa pools), are using water in the temperature range that encourages *Legionella* growth. In addition, these water systems can potentially produce aerosols, increasing the spread of the bacteria. The study also found that *Legionella* were most commonly isolated at temperatures between 35 ºC and 45 ºC, with the greatest increase in viable counts occurring between 37 ºC and 42 ºC. As the temperature falls below 37 ºC, the bacteria's reproductive rate decreases and there is little or no increase in numbers of bacteria below 20 ºC. Temperature is one condition that affects the motility, piliation, and virulence of *L. pneumophila* cultured in bacteriological medium. *Legionella* can survive for long periods at low temperatures and then proliferate when the temperature increases, if other conditions allow (WHO, 2007).

2.5.6.3.2 Symbiotic Microorganisms and Biofilm Influence

Biofilm is a matrix composed by heterogeneous aggregates of bacteria, fungi, protozoa, and algae embedded into extracellular polymeric substances. Drinking water distribution system (DWDS) may have both primary pathogens and oppurtunistic ones (Li *et al.,* 2015). *Legionella* proliferation is enhanced by symbiotic relationships with other microorganisms found in sediment, biofilms in water distribution system. Microorganisms, including *L. pneumophila*, form biofilms as a mechanism to withstand adverse conditions, such as limited nutrients or temperature extremes (WHO, 2007). Biofilms stimulate the growth of commensal microflora, which in turn stimulates the growth of *Legionella* and others in the water distribution system. Biofilms provide a possible habitat for hygienically relevant microbes and have shown a contributory factor in supporting the growth of *Legionella* within potable water environments (Flemming *et al*., 2002).

Legionella grow symbiotically with aquatic bacteria attached to the surface of biofilms and the relationships of *Legionella* with certain algae and bacteria in biofilms foster the growth of *Legionella*, presumably due to the increased availability of nutrients and resistance to disinfection (USEPA, 1999). Moreover, *Legionella* biofilms are better protected from hydraulic shocks and from the action of antibiotics, disinfectants and UV radiation than suspended cells (Manuel *et al*., 2010).

Legionella in the biofilm get suitable condition for survival and multiplication resulting contamination of the water by oppurtunistic pathogen besides detoriration of the water quality. Infection may occur when susceptable group of the population exposed (Cervia *et al*.,2008; Felfoldi *et al*., 2010). Bacterial regrowth in water distribution systems can occur because of the accidental entry of microorganisms at cross connections and broken pipes and

the recovery of microorganism populations affected by disinfectants in water treatment plants (Lee, 2013). Hence, water distribution system in health care facilities, resorts and others require special attention, since it can be a source or reservoir of pathogens and contaminated water can lead to outbreaks and severe infections. Those infections related to biofilms often correlated with resistance of microorganisms to various treatments (Capelletti *et al*., 2016).

So, it is important to control the level of heterotrophic bacteria (HPC), biofilm constituents of the water systems of buildings used by individuals at particular risk to the effects of *Legionella* exposure (Solimini *et al*. 2014; USEPA, 1999). Other factors influencing the survival of *Legionella* in the environment include sediment accumulation, metal content and are usually amplified by ideal water temperature or coexisting environmental factors (USEPA, 2001).

2.6 Diagnosis and Treatment

2.6.1 Diagnosis

Although diagnostic methods have improved since *L. pneumophila* was first described in 1976, no currently available test is able to diagnose all *Legionella spp*. in a timely fashion with a high degree of sensitivity and specificity. Most of the data are applicable to *L. pneumophila*, since sensitivity and specificity estimates for non-pneumophila species are not known (Diederen, 2007). Specimen types, diagnostic tests, and anatomical locations for determining a potential current or recent *Legionella* infection are outlined by Mercante and Winchell (2015), in Figure 8. Some assays are applicable to multiple specimen types, such as culture and nucleic acid amplification.

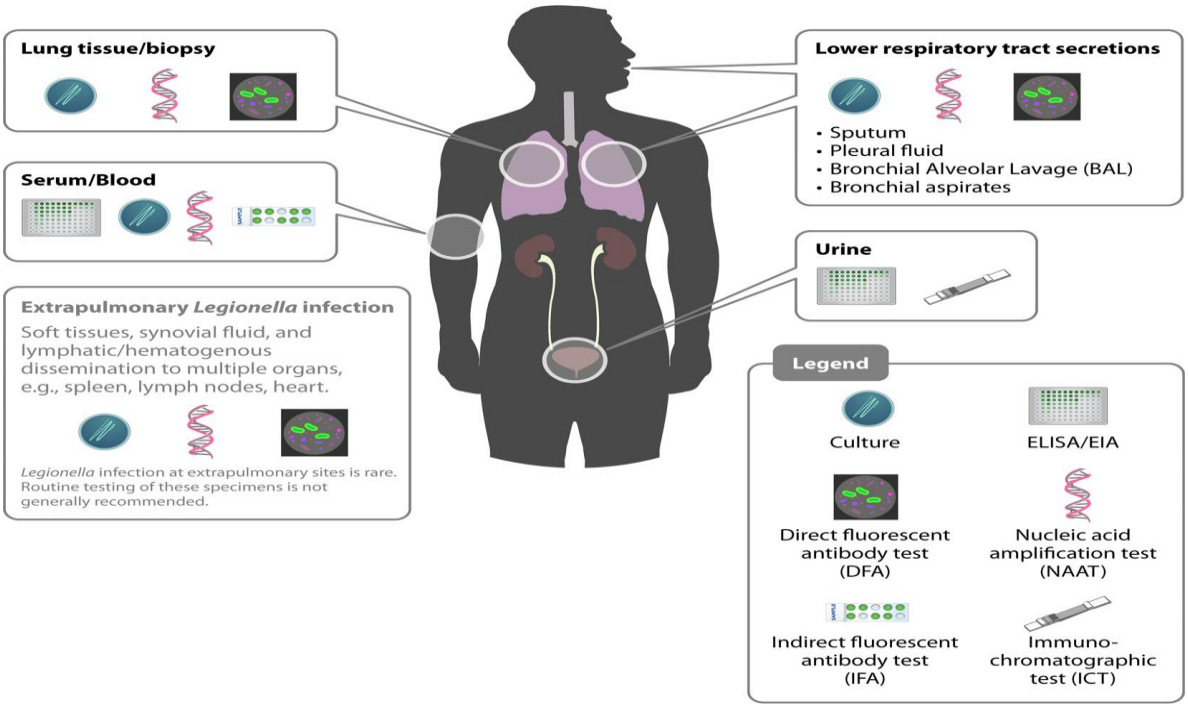


Figure 8 Specimen types, diagnostic tests & anatomical locations for determining *LG* infection

Source: Mercante and Winchell, (2015)

In general, the success of detecting *Legionella* is dependent on the severity of disease, specimen integrity, technical proficiency of the laboratory personnel, and the particular test characteristics. Additional recent emerging methods and technologies may also be used, such as mass spectrometry, but they may not be widely available or accessible. Note that *Legionella* infection at extra pulmonary sites, such as soft tissues or organs (e.g., spleen and heart), is rare (Mercante and Winchell 2015). The sensitivity, specificity and limitations of the available diagnostic tools for *Legionella* which is adopted from Wang *et al.*, (2012) and Jalila *et al*., (2012) are presented on Table 2.

Table 2 The characteristics and limitations of clinical diagnosis for LD.

Tests	Sensitivity (%)	Specificity (%)	Time for testing	Limitations
Direct fluorescent antibody staining	25-70	95	2-4 hours	Sensitivity may change with type of specimen. A false-positive result may occur. The testing results may be affected by skills and experience on the tests.
Culture	＜10-80	100	3-7 days	The quality of sputum specimens is difficult to be controlled. The amount of bacteria in sputum varies with disease progression. The testing results may be affected by skills and experience on the tests.
Urinary antigen test[1]	70-90	>99	15 min-3 hours	This test is used only *for Legionella pneumophila* serogroup type 1 strain.
Serological test	60-80	>95	1-10 weeks	The results based on four-fold increase in antibody titer is just used for retrospective diagnosis that provides only a small benefit for patient treatment in early stage of the disease.
Nucleic acid detection[2] PCR	80-100	>90	Within 4 hours	This may produce un clarified false-positive results. No commercially available assay for testing clinical samples; detects all species and serogroups

Notes:

1. *The similar method was developed for applying to non-urinary specimen although its efficiency needs to be evaluated and recognized. However, no commercial kits for detection of urinary antigen from Legionella pneumophila non-serogroup type 1strain have been developed.*
2. *The data provided here is appropriate for respiratory tract specimen only. Although this method is also applied to urine and serum specimen, the sensitivity is ranged from 30-80%.*

2.6.1.1 Biochemical test identification

2.6.1.1.1 Culture

Isolation of *Legionella spp*., which has a specificity of 100%, is considered the gold standard for diagnosis of LD (USEPA, 1999). A culture isolate is required for further epidemiological typing or for susceptibility testing. Culture diagnosis requires special media, adequate processing of specimens, and technical expertise. Specifically formulated media (most frequently Buffered Charcoal Yeast-Extract media) are required to enhance the growth of

Legionella spp. and suppress other respiratory bacteria. Patients with Legionnaires' disease are often non- productive of sputum and therefore require invasive procedures to obtain respiratory samples (e.g. Sputa, broncho alveolar lavage (BAL), and lung biopsy). The culture method requires several days to obtain a positive result, with most Legionella spp. colonies being detected within 7 days (USEPA, 1999). Species other than *L. pneumophila* may grow at a slower rate and may therefore be detectable only after 10 days of incubation. In addition, some *Legionella spp*. have unusual colony morphology and may therefore be more easily overlooked (Mercante and Winchell, 2015; Blyth *et al.*, 2009; Diederen, 2007).

2.6.1.1.2 Serology

Serological testing for *Legionella* infection is a valuable epidemiological tool but is of less immediate benefit to physicians because of delayed sero-conversion. The indirect Immunofluorescence Assay (IFA) was used to detect antibodies in patients from the Philadelphia outbreak and was instrumental in determining the cause of the illnesses (Vergis *et al.*, 2000). Since then, a number of serologic test methodologies have been developed to detect antibodies to *Legionella spp*., among the various antibody detection methods that are available, IFA and Eenzyme-Linked ImmunoSorbent Assays (ELISA) are the most commonly applied. Immunofluorescence Assay (IFA) remains the standard reference test and is validated for *L. pneumophila and L. longbeachae*. ELISA assays are designed to provide a sensitive screen for Legionellosis and detect IgM using *L. pneumophila* serogroup 1 or *L. longbeachae* sonicated whole cells as antigens. Nowadays, ELISA assays are preferred by many laboratories over IFA testing because they are less subjective, thought to be more accurate than IFA testing and have the potential for automated performance (Mercante and Winchell, 2015; Blyth *et al*., 2009; Diederen, 2007; Vergis *et al*., 2000; USEPA, 1999)

2.6.1.1.3 Detection of *Legionella* Antigen in Urine

The detection of *Legionella* antigenuria has been used already, shortly after the first outbreak in Philadelphia. The antigen detected is a component of the *Legionella* cell wall. Antigenuria can be detected as early as 1 day after the onset of symptoms and can persist for months despite therapy. Popular formats include the enzyme immuno-assay (EIA) and ICT /immunochromogenic test (Mercante and Winchell, 2015; Blyth *et al*., 2009; Diederen, 2007; USEPA, 1999).

2.6.1.1.4 Fluorescent Microscopy

Direct fluorescent-antibody (DFA) staining is a rapid method of directly detecting *Legionella spp.* in respiratory secretions and tissue samples. Although rapid, it is insensitive, requiring large organism numbers for visualization (i.e. severe disease). Reported sensitivity of fluorescent microscopy varies but is consistently less than that of culture. Furthermore, it is technically demanding, requiring experienced laboratory personnel. False positive results may occur because of cross-reactions with other bacteria and yeasts. Problems with both sensitivity and specificity have limited the use of DFA staining in most laboratories (Blyth *et al.*, 2009; USEPA, 1999).

2.6.1.1.5 Polymerase Chain Reaction/PCR

PCR-based detection of *Legionella* DNA in sputum, urine and blood has been described. PCR amplifies minute amounts of *Legionella* DNA, providing results within a short time and enabling detection of infection caused by all *Legionella species* and sero-groups (AWT, 2019). Molecular methods can be formulated to incorporate real-time or multiplex formats. Quantitative real time PCR (qPCR) method can be used for isolation of *Legionella* from

environment and the method is faster sensitive and specific detection. The method can able to detect including dead and viable but not culture able (VBNC) *Legionella.* In this method viable organism that may be able to cause infection cannot be differentiated from nonviable (AWT, 2019) and may result over estimations of infectious risks (Jalila *et al*., 2012; Viscogliosi *et al*., 2009; USEPA, 1999).

2.6.2 Treatment of Legionnaires Disease

Many antibiotics that are usually effective against pneumonia are ineffective against *Legionella* because antibiotics do not enter the respiratory tract cells or alveolar macrophages (WHO, 2007). *Legionella* are susceptible to a wide range of antimicrobial agents when it is extracellular. The first choices for treatment of *Legionella* infections are Erythromycin and quinolone antibiotics (AWT, 2019).

Erythromycin (a macrolide antibiotic) and newer macrolides (e.g., azithromycin) are available that exhibit superior activity to *Legionella* and greater intracellular penetration with potentially fewer adverse effects compared to erythromycin. With development of intravenous formulations, these newer macrolides may be preferred choice of treatment. Moreover for immunosuppressed and transplant patients Quinolones (Ciprofloxacin, Levofloxacin, Moxifloxacin, Gemifloxacin, Trovofloxacin) have shown greater activity against *Legionella* species with higher intracellular penetration than the macrolides (WHO, 2007).

Other antibiotics like tetracycline's (e.g., doxycycline, minocycline, and tetracycline) and the combination of trimethoprim and sulfamethoxazole have shown variable success to *Legionella* treatment. Intravenous treatment is the preferable way of treatment for infected individuals but oral therapy can replace after fever diminishes (USEPA, 2001).

2.7 Prevention and Control

There is extensive evidence that inadequate management of water distribution systems has led to outbreaks of illness in both developed and developing countries. Management of distribution systems of manmade water system often receives too little attention. Distribution systems can incorrectly be viewed as passive systems with the only requirement being to transport water from the outlets of treatment plants or source to consumers. The most effective means of ensuring the safety of water supplies is, through the use of a comprehensive risk assessment and risk management approach incorporated in a water safety plan (WSP).

WSP applies to all steps of a water supply, from source to point of use. Normal practice is to develop an integrated WSP applying to all components, from catchment through treatment and distribution (WHO, 2014). The WSP approach is based on identifying all significant risks to public health, ensuring that effective controls and barriers are applied to minimize these risks to acceptable levels, and monitoring the operation of the controls and barriers to ensure that safety is maintained (WHO, 2011).

Besides that of WSP controlling *Legionella* in particular and other waterborne pathogens in general, at least two strategies that are most appropriate and cost-effective can be practiced. The first approach is based on periodic, routine culturing of water samples from the potable water system for the purpose of detecting *Legionella spp*. The second approach to preventing and controlling the risk factors for prevalence of legionellosis from prevailing in the water supply system. For instance, for the purpose of controlling it is advisable to look for both *HPC* (heterotrophic plate count) and metal constituents of the water systems of buildings to determine *Legionella* exposure (Solimini *et al.,* 2014; Jalila *et al*., 2012).

Otherwise *Legionella* and other water borne pathogens continue to be a major threat to humans despite scientific and technological advances. Hence, the control and prevention of *Legionella* and other waterborne pathogens need a competent management attention based on a correct risk assessment (USEPA, 1999).

Hence, countries should have proper framework for safe water supply to humans. This is from the reason that the continuous delivery of safe water requires effective management and operation throughout the water-supply chain starting from catchments to consumer points of use. If *Legionella* and other heterotrophic bacteria are observed, prevention can be accomplished by disinfection of water distribution system. For this, the conventional plate culture method described in the standard International Organization for Standardization (ISO) 11731-2-2004 is a very important technique for the detection of *Legionella spp.* Most developed countries have their own monitoring standards for *Legionella spp.* The health-based targets for *Legionella* in piped water systems for some countries are presented in Table 3.

Table 3 Examples of health-based targets for *Legionella* in piped water systems

Country	Value (CFU/liter)	Comment
France	<1000	Target for general public facilities
	<100	Target for prevention of nosocomial infections
	<50	Target where at risk patients are hospitalized
Germany	1000	
The Netherlands	100	Guideline target
United Kingdom	<100	Guideline target

Source: WHO, (2007)

After detection of unacceptable high levels of *Legionella*, effective decontamination and maintenance of water system are critical for prevention of outbreaks of legionellosis. Some of the methods used to disinfect water distribution system include thermal (super heat and flush), hyperchlorination, copper-silver ionization, ultraviolet light sterilization, Ozonation, and instantaneous steam heating systems (WHO, 2014; Jalila et *al*., 2012; USEPA, 1999).

Each of the proposed management options has their own advantages and dis advantages. If possible, application of combined management options has great efficiency in managing *Legionella.* Some of *Legionella* management options as depicted in Table 4 below.

Table 4 Management methods in water distribution system

Method	Advantage	Disadvantage
Thermal Eradication ***Super heat & Flush***	• used in an outbreak situation	• Potential for scalding, re-colonization • require considerable energy
Silver/Copper ***Ionization***:	• Used to hot-water distribution system	• Silver and copper ions are not allowed for disinfection of drinking water in each country. • Silver ions may cause corrosion of the system and, • Electrodes or the silver solution have to be replaced periodically
Hyper-chlorination:	• Eradicate completely from the water system • valid-term option in old buildings • requires precise monitoring of the chlorine level and personnel	• Chlorine has no effect on *Legionella* in blind pipes, • Over time it has a corrosive effect on pipe. • Cost for installation of a chlorinator • Cause human health problems. trihalomethanes
Ultraviolet light ***Irradiation :***	• eradicates without the addition of chemicals to the water • positioned to disinfect the incoming water	• UV lamps are susceptible to scale and sediment deposits. • Concomitant use of bacteriological filters is recommended Cost
Ozone:	• Eradicates & destroys the biofilm throughout the system. • not having to handle chemicals	• Price of aerial oxygen by corona discharge and injected

Source: Qadreyah *et al*., (2012); Jalila *et al.*, (2012); USEPA, (1999).

As chlorine is one of known disinfectant in water distribution system, for its effectively in disinfecting regime, other physical parameters should have to be properly managed side by side. Institutions, hospitals that have complex water distribution to use automated chlorinator to manage the possible contamination in water system. But this is not the case in most developing countries on which most uses the coming treated water as it is. Table 5 outlines technologies that provide sufficient residual chlorine level for controlling *Legionella* from water distribution system.

Table 5 Technological options providing residual disinfection to control *Legionella spp*

	Supplemental chlorine	Chlorine Dioxide	Monochloramine	Copper Silver Ionization
Typical concentration	2-4mg/l, free residual chlorine	0.1-0.8mg/l ClO_2	2.0-4.mg/l as Cl_2	Copper 0.2-0.8mg/l Silver 0.02-0.08mg/l
Distribution system residual	Yes	Yes	Yes	Yes
pH	pH>8 affects efficiency	No impact in 6-10.0 range	No impact in 7-9 pH range	pH> 8.5 may affect efficiency
T^o	Elevated temperature accelerates decay	Elevated temperature accelerates decay	Minimal impact by elevated temperature	No impact by temperature
Disinfection by products	THM &HAAS	Chlorite	Reduced THM & HAAS compared with chlorine	No Chemical reaction to form by products
Drinking water standard	Chlorine <4mg/l	Chlorine dioxide <0.8mg/l	Chlorinamine <4mg/l as Cl_2	Copper <1.3mg/l Silver <0.10mg/l

Notes: *Cl_2= Chlorine, ClO_2= Chlorine dioxide, HAAS= Halo Acetic Acids, THM=Tri Halo Methane* **.** Source: Sidari *et al*., (2014)

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Study Design

A cross-sectional purposive descriptive study which entails consecutive sampling of water from hot springs, natural water bodies and health care water facilities distribution system was conducted from *Dec 14, 2016 to June 20, 2018 in Ethiopia.*

3.2 Description of the Study Area

3.2.1 Study Area

The study area was located in one city administration (Addis Ababa City administration) and three regional states (Amhara, Oromiya and Southern Nations Nationalities Peoples Regional states (SNNPRs). Purposive sampling was used as main sampling techniques to select Hot springs and natural water body's sites by considering location, population density cost, time, laboratory facilities and other elements. However sample sites of hospital and water distribution system selected randomly. The overall sampling points are presented in Figure 9 &10.

3.2.1.1 Natural water bodies

Ethiopia is gifted with a variety of freshwater aquatic ecosystems, including Lakes, rivers wetlands which have significance in socio-economic, political and scientific importance. The need for water for domestic purpose, urbanization and industrialization is strongly ascending and complex. Natural water resources are vital resource for the developmental activities in

both agricultural and industrial sectors in one hand and population growth on another. *Legionella* isolation was done from purposely selected natural water bodies of Lakes of Hora (LHO), Babogaya (LBA), Hawassa (LHA) are found in the main Rift Valley region of Ethiopia (Rift Valley Lakes). Except two lakes Lake Hawassa (LHA) and Lake Tana (LTA), all sampled natural water bodies are found in the Oromiya regional state. LTA is found outside the rift valley region in the Amhara regional state. But, LHA is found in Rift Valley in Southern Nations Nationalities Peoples Regional state (SNNPRs). Some of the morphometric and physical feature of sampled natural water bodies are presented in Table 6.

Table 6 Morphometric and physical features of the Ethiopian Lakes.

Lakes	Altitude (m) ASL	Surface area (Km^2)	Max depth (m)	Mean depth (m)	Catchment (Km^2)	Ref**
Hawassa	1680	80-90	22	11	1250-1455	1&2
Tana	1775	3156	14.1	9	16500	1
Hora Arsedi	1850	1.03	38	17.5	-	1
Babogaya	1870	0.58	65	38	-	1

NB ** 1= Zinabu *et al.,* 2002; 2= Tenalem, 2009; ASL= Above Sea Level

3.2.1.2 Hot spring/Spa Water Facilities

To detect the prevalence of *Legionella* four Hot springs that are found nearby of Addis Ababa, publicly known, relatively have access for public transport were purposely selected. Hence one hot spring from Addis Ababa, One from SNNPRS and two from Oromiya regions selected.

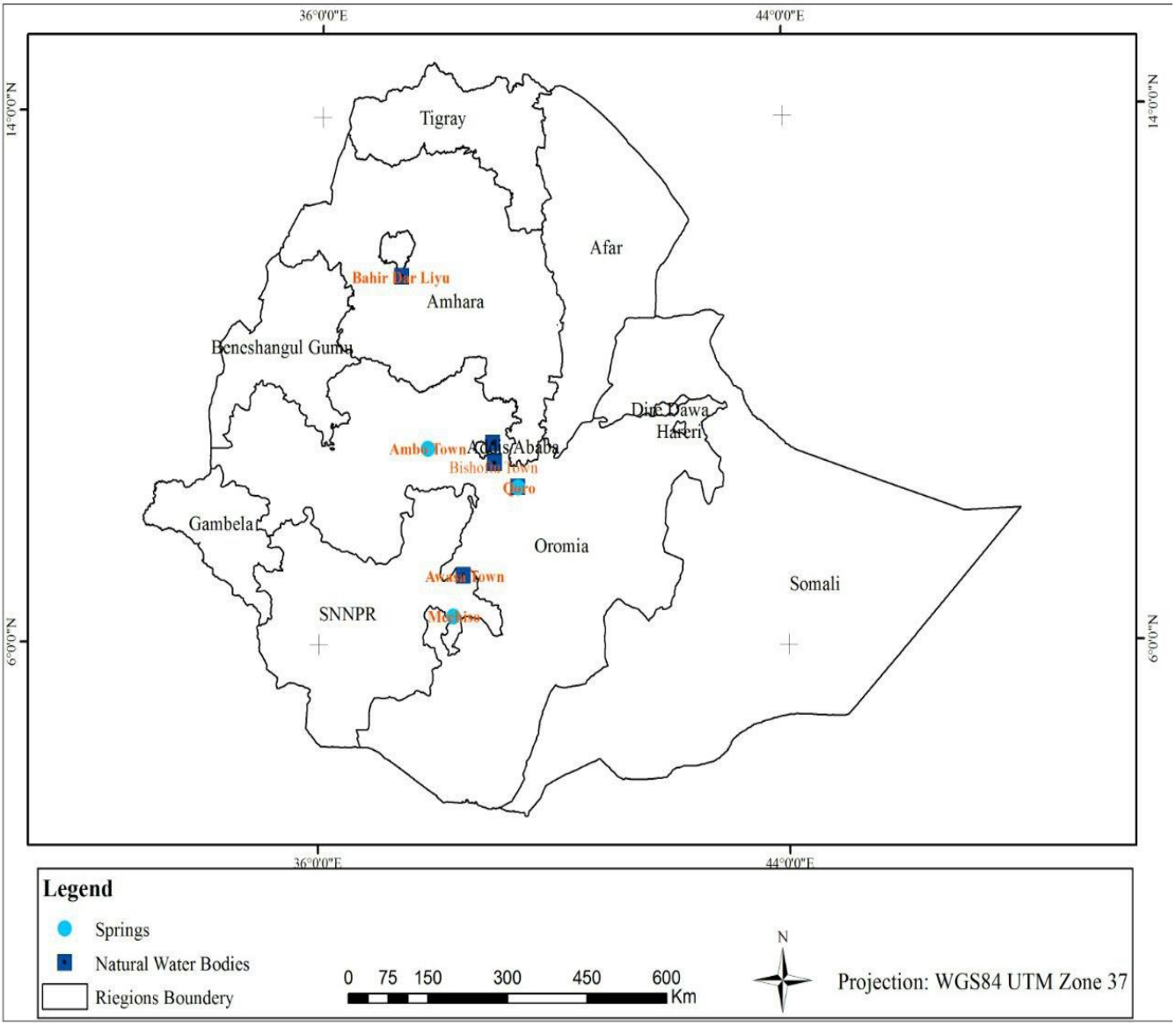


Figure 9 Sampling point of Hot Spring and Natural water bodies

3.2.1.3 Hospital Water Distribution System

The available hot and cold water samples together with the biofilm from hospital water distribution system were collected from two randomly selected referral hospitals from Addis Ababa. These were Yekatit 12 hospital medical college and Zewditu hospital. Yekatit 12 Hospital Medical College is found at “Arada” sub city, district 06 of Addis Ababa. The

hospital was established in 1922 GC and located nearby of Yekatit 12 memorial square ("Sidist Kilo") South of the AAU main campus and West of the Federal Supreme Court. The hospital has giving services in newly completed complex building. Zewditu Hospital is found at "Kirkos" sub city district 07 of Addis Ababa. The hospital is located nearby of Addis Ababa Spa, ("FileWuha"), Ambassador Theater and National Palace. Both hospitals are providing comprehensive medical services as referral teaching Hospital and managed by Addis Ababa City Administrative Health Bureau.

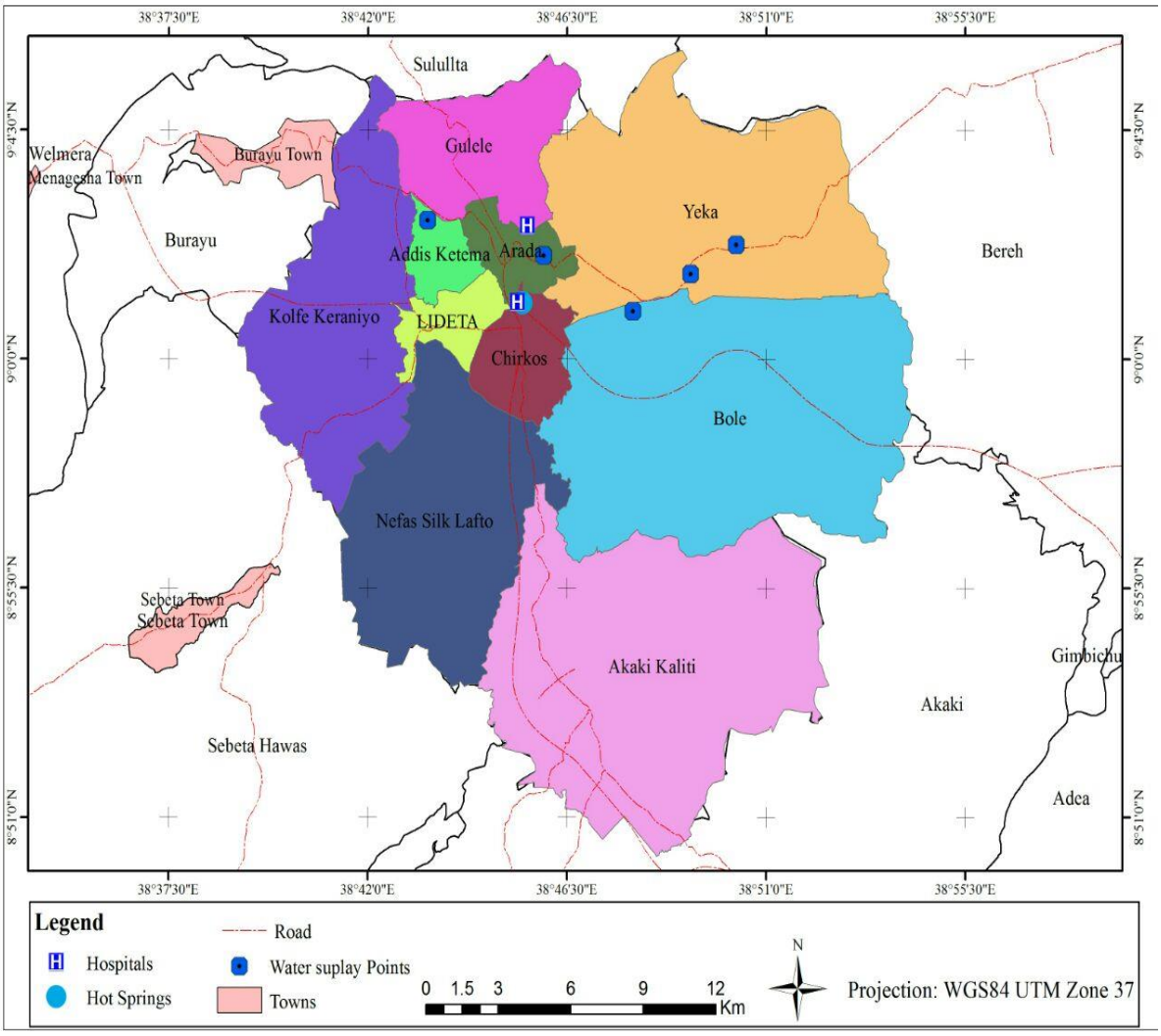


Figure 10 Water sampling points found at Addis Ababa

3.2.1.4 Addis Ababa Water distribution System

Modern potable water distribution started during the era of Emperor Minilik II (1900) in Ethiopia. The first distribution line constructed after Addis Ababa became the capital city of Ethiopia which was from “Entoto Kidan Mihiret” area towards “Arat kilo” where the Palace is found. Due to urbanization and economic growth in Addis the demand of water supply is increasing and an authority Addis Ababa Water and Sewerage Authority (AAWSA) organized under proclamation number 68/1963 and 10/87 to provide water and sewerage service to needy people in Addis Ababa. Currently the AAWDS has more than 480,000 registered potable water customers. From the surface and ground water, the AAWSA produces potable water about 608,000m^3 per day. The main sources of this potable water are “Gefersa” dam reservoir and treatment plant about 30,000m^3 / day (4.93%), “Legedadi” dam reservoir and treatment plant about 195, 000m^3 /day (32.1%) and the remaining 383,000m^3/day (63%) from ground water source from different parts of Addis Ababa. The main drinking water sources in Addis Ababa presented in Figure 11.

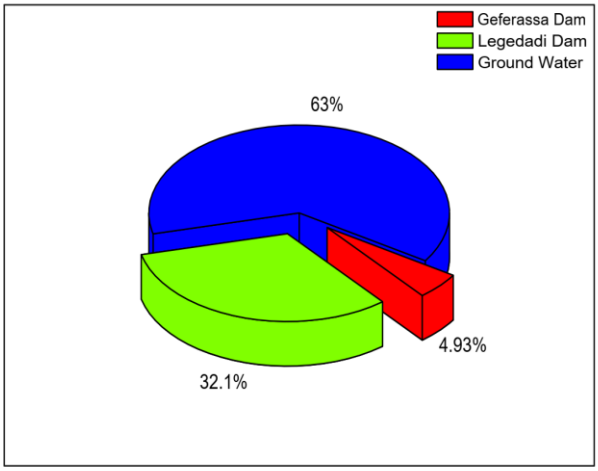


Figure 11 Main Drinking water Sources Addis Ababa City

Table 7 Geographical Position System Sampling points

S/N	Sample site	Latitude (N)	Longitude (E)	Altitude	S/N	Sample site	Latitude (N)	Longitude (E)	Altitude
1	L. Tana	$11^0$35’52.36’’	$37^0$23’17.77’’	1789	10	HS4	$8^0$23’45.824’’	$39^0$23’51.214’’	1360
2	L. Hawassa	$7^0$03’16.50’’	$38^0$27’56.69’’	1689	11	HS2	$8^0$58’51.50’’	$37^0$51’12.24’’	2099
3	L Hora Arsedie	$8^0$45’56.48’’	$38^0$59’44.41’’	1873	12	HS1	$9^0$01’06.20’’	$38^0$45’30.22’’	2359
4	L. Babogaya	$8^0$47’20.33’’	$38^0$59’49.42’’	1882	13	HS3	$6^0$26’09.46’’	$38^0$17’25.21’’	1413
5	AAWDS	$9^0$01’40.47”	$38^0$49’17.41’’	2420	14	Awash R	$8^0$23’59.021’’	$39^0$23’43.814’’	1350
6	ZH	$9^0$01’06.76’’	$38^0$45’22.43’’	2357	15	Legedadi Dam R	$9^0$04’06.93’’	$38^0$57’41.57’’	2456
7	Y12 H	$9^0$02’36.92”	$38^0$45’36.00’’	2491	16	AAWDS	$9^0$02’44.02’’	$38^0$43’20.99’’	2508
8	AAWDS	$9^0$02’14.99”	$38^0$50’19.27’’	2471	17	AAWDS	$9^0$00’56.81’’	$38^0$47’59.13’’	2370
9	AAWDS	$9^0$02’02.23’’	$38^0$45’58.16’’	2447					

3.3 Methodologies

3.3.1 Culturing of *Legionella*

3.3.1.1 Sample Collection and Transporting

Potential sample sites that could produce aerosols were primary chosen from each water distribution system. Then the available hot and cold water samples collected from drinking water distribution system, hospitals and hot springs collected mainly from the bathroom outlets (shower heads or bathroom tap). Sampling locations were randomly selected and triplicate water samples collected within two weeks intervals. From water distribution systems sample types, one biofilm swab and one bulk water sample were collected from each sampling site (i.e. each showerhead or faucet) (CDC, 2015). All Sample collection bottles and test tubes cleansed and rinsed carefully with distilled water, and sterilized before collection of any samples.

Biofilm samples: - Biofilm samples from Water distribution system (WDS) were collected before bulk water sample collected by removing the aerator or shower head after brief flow time. Swab samples were collected by sterilized Dacron Tipped swabs by rubbing rotating the swab three to four times inside water pipe. Then the collected swab samples were kept in 10-15mL capacity sterilized screw cupped test tubes. About 3-5mL of water from the same faucet added to the test tubes to moisten the swab samples while transporting. The swab stem snapped approximately one inch from the top of the tube and a drop of 0.1N sodium thiosulfate solution added to the tubes to neutralize residual disinfectants (only for potable water systems). Proper labeling of samples date, time and location was done before transporting the samples for further microbial analysis (CDC, 2015 and CDC, 2005).

Bulk water sample: - Bulk water samples from water distribution systems collected after the biofilm swab samples collected. Bulk water samples collected after a brief flow time until the water is warm but not hot (for the hot water sample). About one litter of water sample was collected by pre sterilized 1000mL capacity bottles leaving approximately one inch space at the top of sample collecting bottles. In case, if potable water samples treated with Chlorine, samples were collected by a sterile bottle that contains Sodium thiosulfate (bottles sterilized after adding 0.5 mL of 0.1 N or 3% sodium thiosulfate per litter) which helps to neutralize residual disinfectants.

Only bulk water samples were collected from selected surface waters (Lakes, River, and Reservoir). Except from the river and reservoir, water samples from all of the Lakes were taken with pre-sterilized sampling bottles from positions one to three meters away from shore station with the help of available swimmers and or boat driver. For the river and the reservoir samples collected by sterilized plastic bottles from near the shore or the littoral zone.

Some In-situ sample analysis for physical parameters were done while collecting biofilm and bulk water samples. Cold water samples collected and analyzed after brief flow before the hot water samples. Hot water samples collected after running the hot water until it is as hot as it will get. About 100–300 mL water sample collected by plastic sampling bottle and level of temperature, pH, Total Dissolved Solids (TDS), Electrical Conductivity (EC) and Free residual Chlorine(if any) measured while the others water chemical parameters measured ex-situ after the samples reached to laboratory within 4–8 h of sample collection.

Transporting :- All collected samples transported to the AAWSA (Addis Ababa Water Sewerage Authority), microbiology laboratory by keeping samples at <4°C in ice box and analyzed within 6-8 hours of collection for bacteriological and physicochemical examination; in case where analyses could not begin within 6 hours, samples were preserved and kept at <4°C and processed within 24 hours.

3.3.1.2 Microbiological Analysis

Culture method was used as golden standard method for *Legonella Spp* detection as described by Phin *et al*., (2014). Buffered Charcoal Yeast Extract Agar base ISO 11731-2 (BCYE Agar Base) with *Legionella* GVPC supplements) was used as selective culture media for *Legionella*.

3.3.1.3 Water Samples preparations and Concentration

Filtration: - For estimated Low-bacterial-count in water: water samples concentrated by filtration. The collected one litter water sample concentrated by membrane filtration technique by passing the water sample aseptically through 47 mm diameter cellulose type HA membranes having a pore size of 0.2 μm Isopore ^{TM} GTTP Millipore Membrane filters, (Merk Millipore Ltd. Tullagreen, Carrigtwohill, Co, Cork, IRL, Ireland) membrane filters by negative pressure. Then the filtered membrane has been removed aseptically from the membrane filter holder with sterile filter forceps, folded to the outside and transferred to 10 mL capacity sterile screw capped culture tubes containing 5ml of sterile distilled water.

Direct plating (non-potable water):- Non-potable water (samples from rivers, Lakes) rarely requires concentration and processed directly. Non-potable water sources generally require a form of heat or acid treatment to reduce the presence of non-*Legionella* organisms present in the sample. In present study non potable water samples treated with heat only by using water bath at 50°C for about 30 minutes. Samples that showed high concentrations of bacteria upon plating, further treated with serial dilutions with sterile distilled water. Mainly 10 mL bulk water sample diluted with 10 mL sterile distilled water in a >25mL centrifuge tube (APHA, 1998).

Sample dispersion: - Microorganisms dispersed or eluted from the membrane filter or aggregates by mixing with a vortex mixer (3 × 30 s). Similarly Isolation of microbes from the collected swab samples were done after the samples were vortex mixed for about 1:30 minutes. According to APHA, (1998), the maximum elapsed time between collection and analysis of microbial samples is 8 hs (maximum transit time 6 h, maximum processing time 2

h). When analysis cannot begin within 8 h, the samples maintained at a temperature below 4°C (without freezing) and analysis done within 24 h of sample collection time.

Figure 12 Some of Laboratory instruments used for water quality analysis

3.3.1.4 Standard culture method for *Legionella Spp* isolation

The culturing of *Legionella spp* was performed by spread plating an inoculum onto buffered charcoal yeast extract (BCYEα). Aliquots of 0.1 mL of the concentrated specimens from bulk water and biofilm swab samples were inoculated in duplicates using spread plate technique on

modified BCYE agar (SIGMA-ALDRICH 78123 Buffered Charcoal Yeast Extract Agar base ISO 11731-2 BCYE Agar Base with *Legionella* GVPC supplements).

Buffered charcoal yeast extract (BCYEα) is comprised of *Legionella* yeast extract (YE)10g/L Activated Charcoal 2mg/L agar base supplemented with *Legionella* BCYE growth supplement ACES buffer N-(2-acetamido)-2-aminothane-sulfonic acid) 10g/L; alpha-Ketoglutarate, monopotassium salt 1g/L; 1.0 g/L, ferric pyrophosphate (125mg/L); L-cysteine HCL (200mg/L) and Agar (12g/L). In addition, antibiotics were added to the medium to prevent the growth of *non-Legionella* microorganisms along with *Legionella* selective supplement containing ammonia free glycine (1.5 g/L), vancomycin (0.5 mg/L), polymyxin B SO4 (39600 IU) and cycloheximide (40.0 mg/L) (GVPC)] (CDC, 2005).

Cultures were incubated under microaerophilic conditions in a candle jar at 35°C to further reduce the growth of *non-Legionella* organisms. The plates in duplicates were incubated in a humidified environment with at least for at least 5-7 days. After incubation, the plate's colonies were examined under a dissecting microscope and those colonies with structures that are convex and round smooth with entire edges, bright white in color with a textured appearance "cut-glass like" or speckled. The white center of the colony is often bordered with blue, purple, green or red auto fluorescence colonies were counted as suspected colonies.

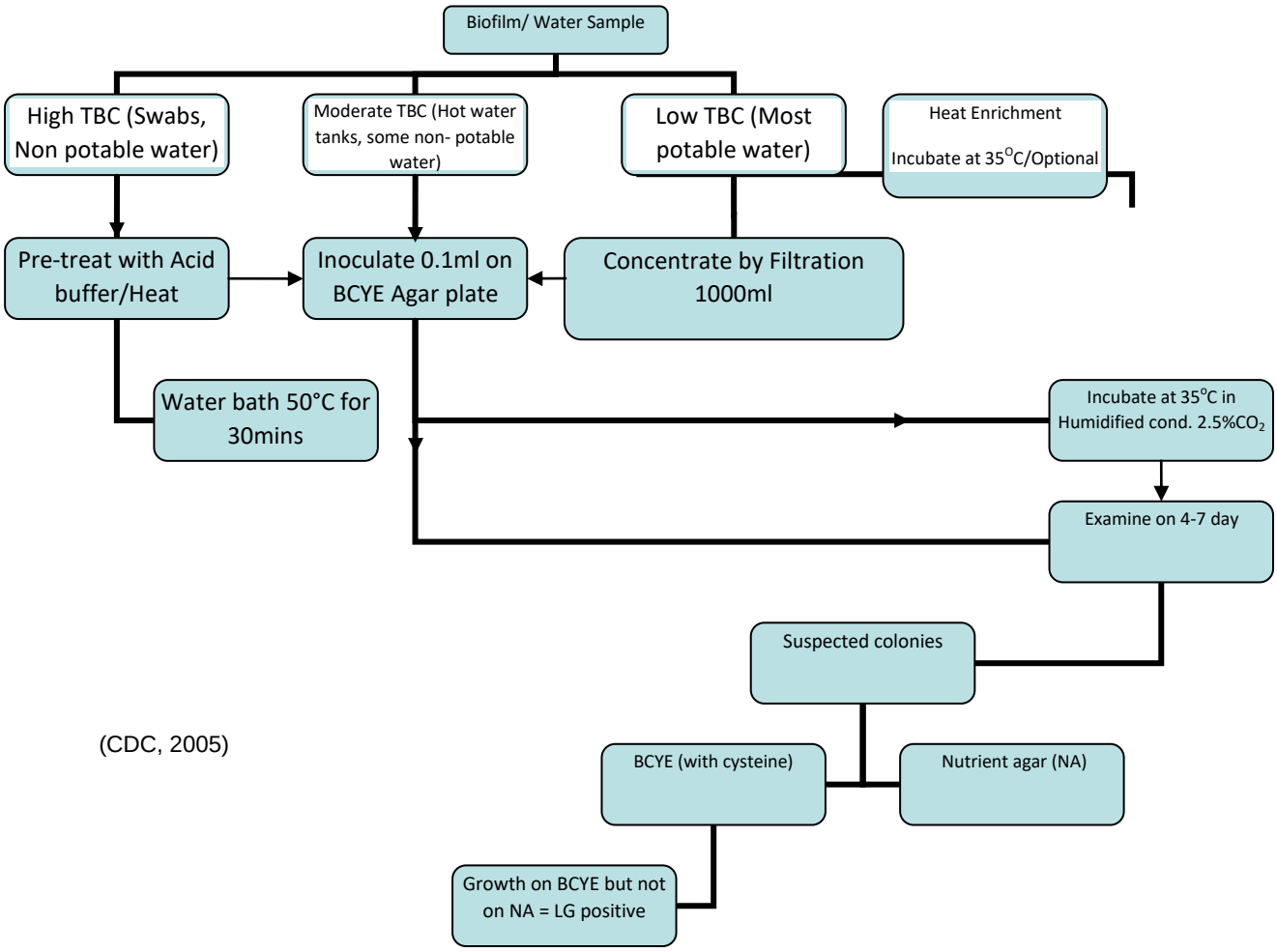
To test cysteine dependency, suspected colonies (at least two) were further seeded or inoculated on to two different culture media namely on Buffered Charcoal Yeast Extract Agar with L-cysteine [BCYE (+)] and BCYE agar plate without L-cysteine [BCYE(-)]. Nutrient agar plates used alternatively for L-cysteine [BCYE (-)] media

Subsequently, BCYE (+)] and Nutrient agar (Oxoid) were used to inoculate colonies and cultures then incubated at $35^{O}C$ for two to three days. In accordance with the recommendations of ISO/DIS 11731-2, colonies that grew on a [BCYE (+)] but not on Nutrient agar were recorded as presumptive *Legionella* species.

Plate showing the highest number of confirmed colonies was used to estimate the number of *Legionella spp* in the original sample. The number bacteria estimated as colony Forming Units (CFU) of *Legionella* species was determined by the following formula per liter in the original un-concentrated sample:

$$C = \frac{n \times V}{I} \times \frac{1}{s}$$ ----------Equation 1

Where: C = CFU /liter in the original sample; n = number of colonies on the plate; v = volume (mL) of the concentrate; i = volume (mL) inoculated onto plate; s = volume (liters) of water from which the micro flora were concentrated (HPA, 2006). Concentrations of *Legionella spp* in water samples are expressed as colony forming units per liter (CFU/L); on the hand the results of biofilm samples were expressed in qualitative terms (*Legionella* found/*Legionella* not found) *Legionella* isolation procedure are presented in Figure 13



(CDC, 2005)

Figure 13 Sampling procedures followed for isolation of *Legionella spp*

3.3.2 *Heterotrophic Plate Count*

Most of the microbial sample collection procedure for *HPC* analysis was performed based on the methods used for the *Legionella*. The *heterotrophic plate count*, formerly known as the standard plate count is a procedure for estimating the number of live heterotrophic bacteria in water and measuring changes during water treatment and distribution. *HPC* test was performed by spread plate method without dilution of the samples (potable water) or after

dilution of samples up to 10^{-3} (non-potable water) especially for those samples from natural water bodies.

Duplicate Plate count Agar (Oxoid) plates prepared for each volume of sample or dilution examined. Samples or and dilution thoroughly mixed by using shaker or vortex mixer for about 15s. After appropriate labeling of sample number, date and the like about 0.1 mL of the samples spread plated on surface by sterile bent glass rod on (65 cm^2) petri dishes. Then after the inoculum absorbed completely into the medium, plates were incubated at 30°C for 48 hrs. Colonies from the agar surface were counted by colony counter and reported as Colony Forming Units per mL (WHO, 2006; APHA, 1998). All microbiological analyses were done by following strict aseptic techniques of microbiology procedures. Average number of bacterial colonies was calculated by

$$\frac{CFU}{mL} = \frac{Colonies\ counted}{Actual\ volume\ of\ sample\ in\ dish,\ (mL)} \qquad \textbf{Equation 2}$$

3.4 Physicochemical Analyses:

Water temperature, pH, Total dissolved solid (TDS) and Electrical Conductivity (EC) were measured by potable TechPro II™ Series Model THP1 (MYRON L® COMPANY USA) instrument during sample collection (In-situ) and residual free chlorine if any by DPD colorimetric method. Other chemical parameters like Potassium, Phosphate, Sulfate, Total alkalinity, Iron and Fluoride were analyzed at the laboratory by using photometer 7100 UK according to Wagtech instructions (Palintest® Photometer systems for water analysis, Wagtech international UK).

Good laboratory quality assurance protocols applied and followed starting from the sample collection to analysis. This includes use of standard operating procedures, calibration of instruments with standards, analysis of reagent blanks and the like.

3.5 Method for Data Entry and Analysis

Data were entered and analyzed using IBM SPSS version 20 Software, Microsoft Excel XP version 2010 and Origin Lab version 6.1 Software was used for data analysis and technical graphics. Logarithmic transformations were used in statistical analyses to normalize the non-normal distributions, and results presented as mean with standard deviation. The bacteriological data converted into Log_{10} (x+1). The results were presented in descriptive statistics using frequency tables, cross tabulation and pie charts. The results of bacteriological and physicochemical analyses were compared with national and WHO guidelines for drinking water. P-value < 0.05 or <0.01 was considered to indicate statistically significant association.

CHAPTER FOUR

4 RESULTS AND DISCUSSIONS

A total of 220 water samples from different water types (Hot springs, Lakes rivers, Drinking water reservoir, Hospital water and Addis Ababa Drinking water distribution system) were collected and analyzed for the prevalence of *Legionella* and associated physicochemical factors. Proportionally 31(14.1%) of the samples were from Lakes and river, 57(25.9%) of the samples were from two hospital water distribution system found in Addis Ababa, 26(11.8%) water samples from Addis Ababa water distribution system and the remaining 106 (48.2%) from the hot springs found in three Ethiopian regions. The proportion of total analyzed water samples by sources or type is presented in Figure 14A. Sampling by area includes 115 samples from Addis Ababa, 73 samples from Oromiya, 27 samples from SNNPRS and Five samples from Amhara regional states Figure 14 B

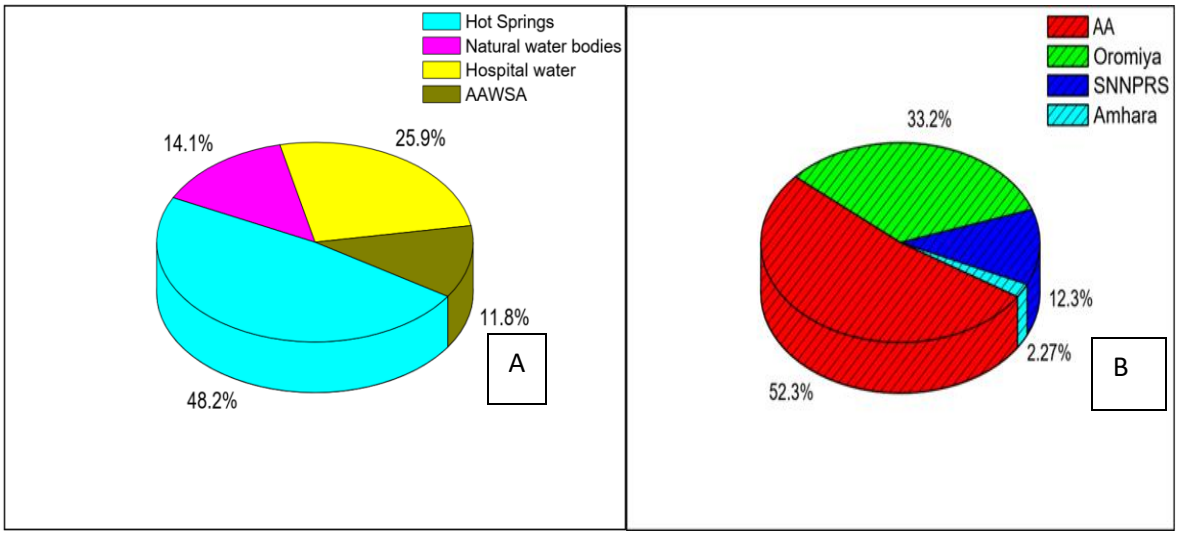


Figure 14 Proportion of analyzed water sampling by Source and area

4.1 Hot Spring Water Samples

A total of 106 samples were analyzed from three regions in Ethiopia. Among these, 32 (30.2%) samples were from Addis Ababa, 23(21.7%) from SNNPRs sates and 51(48.1%) from Oromiya regions. A total of 54 (50.94%) samples were taken from shower tap, 38(35.85%) from biofilm samples and the remaining 14(13.21%) from source of hot spring. *Legionella* were positive from 29(27.4%) of water samples and 17(16%) the biofilm samples.

4.1.1 Physicochemical Quality of Hot Spring Water Samples

The physicochemical water quality of the hot springs is presented in Table 8. Temperature ranges from 31.2 (HS2) to 55.1^{o}C (HS1); the pH range from 6.29 (HS2) to 8.1(HS1). When compared hot springs with potable water, hot springs relatively contain higher level of total dissolved solids(TDS) that ranged from 1099.2(HS2) to 2760.1(HS3) mg/L and mean Electrical conductivity ranged from 1181.3 to 3069.3(HS3)(µS/cm). The Chemical quality of hot springs also found diverse. The Sulfate(SO_4^{2-}) concentration ranged from 1.81(HS1) to 80.3(HS4) mg/L; Iron(Fe^{2+}) level range from 2.60mg/L(HS2) to 55.70 mg/L (HS4); Potassium(K^{+}) from 10.2mg/L (HS1) to 29.5 mg/L(HS2); Phosphate(PO_4^{2-}) from 11.20mg/L (HS1) to 91.4mg/L (HS3); Fluoride(F^{-}) from 1.91mg/L(HS2) to 43.2mg/L(HS1) while Total alkalinity($CaCO_3^{2-}$) from 850.mg/L (HS2) to 5373.0mg/L(HS4). Table 8

Table 8 Mean and Range values of physicochemical parameters of hot springs Ethiopia 2018[a]

		Sampling Sites			
Parameters	Values	HS1 (n=32)	HS2 (n=26)	HS3(n=23)	HS4(n=25)
WT	Mean(SD)	48.3± 5.4	35.3± 1.4	43.6±3.3	41.6±3.9
	Range	34.2-55.4	31.2-36.3	38.7-49.3	35.4-46.5
pH	Mean(SD)	7.5±0.3	6.5±0.2	6.9±0.2	6.9±.3
	Range	7.10-8.10	6.29-7.20	6.40-7.30	6.51-7.52
TDS	Mean (SD)	2433.4±192.7	1151.4±16.5	2569.4±286.4	1463.4±103.1
	Range	1670.1-2659.1	1099.2-1186.1	2017.2-2760.1	1317.1-1640.2
EC	Mean(SD)	3084.1±369.6	1553.1± 79.8	2937.4±112.8	2012.5±168.3
	Range	1850.2-3381.3	1181.3-1591.2	2720.1-3069.3	1753.2-2628.4
SO_4^{2-}	Mean(SD)	7.5±3.6	39.3±4.9	59.7±14.5	50.8±14.5
	Range	1.81-15.42	29.7-59.50	30.30-71.4	29.5-80.3
K^+	Mean(SD)	30.1±15.1	34.9±5.6	18.1±1.4	15.0±2
	Range	10.2-51.1	29.51-55.40	15.41-20.31	11.50-19.40
Fe^{2+}	Mean(SD)	19.1±7.1	6.1±2.3	13.4±1.1	23.1±11.9
	Range	10.91-40.20	2.60-9.60	10.51-14.50	10.90-55.70
PO_4^{2-}	Mean(SD)	24.6±11.6	42.9±12.3	38.2±12.1	20.9±3.3
	Range	11.20-75.1	18.2-68.1	29.70-91.40	15.40-26.61
F^-	Mean(SD)	30.4±7.8	2.6±0.7	14.9±1.1	29.6±7.3
	Range	20.1-43.2	1.91-4.60	13.60-17.3	12.71-37.81
$CaCO_3^{2-}$	Mean(SD)	2072.8± 244.1	1001.9±85.4	1797.4±285.1	4752.0±665.3
	Range	1595-2500	850-1150	1010-2080	3287-5673

[a] *Data reported in mg/L except for pH, EC (µS/cm) and Temperature (^OC);WT=Water Temperature; HS1= Hot spring site 1; HS=: hot spring site 2; HS3= Hot spring site 3;HS4= Hot spring sample site 4*

Among the tested physiscochemcial paramters in the sampled hot springs ccomparatively, higher level of Fluoride measured from HS1 and HS4 while HS2 has lowest level. Highest Flouride level HS1(30.4mg/L) and lowest in HS2 (2.6mg/L). The mean temperature was highest at HS1(48.3^{O}C) and lowest at HS2 (35.3^{O}C). The pH level was highest at HS1(7.5) and lowest HS2(6.5), Poatssium level was highest at HS2 (34.9mg/L) and lowest HS4 (15.0mg/L), sulfate level were highest in HS3 (59.7mg/L) and lowest in HS1(7.5mg/L).

The mean Iron level of hot spring water sample of HS3 was two times higher than HS2 while the mean Iron level of HS1 and HS4 were higher than HS2. The iron level was highest in HS4 (23.1mg/L) and lowest in HS2 (6.1mg/L). phosphate level was highest HS2 (42.9mg/L) and lowest HS4 (20.9mg/L). The mean Alkalinity level of sampled hot springs was 1 to 4 times higher than >600mg/L table 8. It been observed that *Legionella* were isolated from such divers physiscochemicalenvironment of sampled Ethiopian hot psrings. The mean temperature, pH, Iron, Fluoride and others parameter presented in Figure 15.

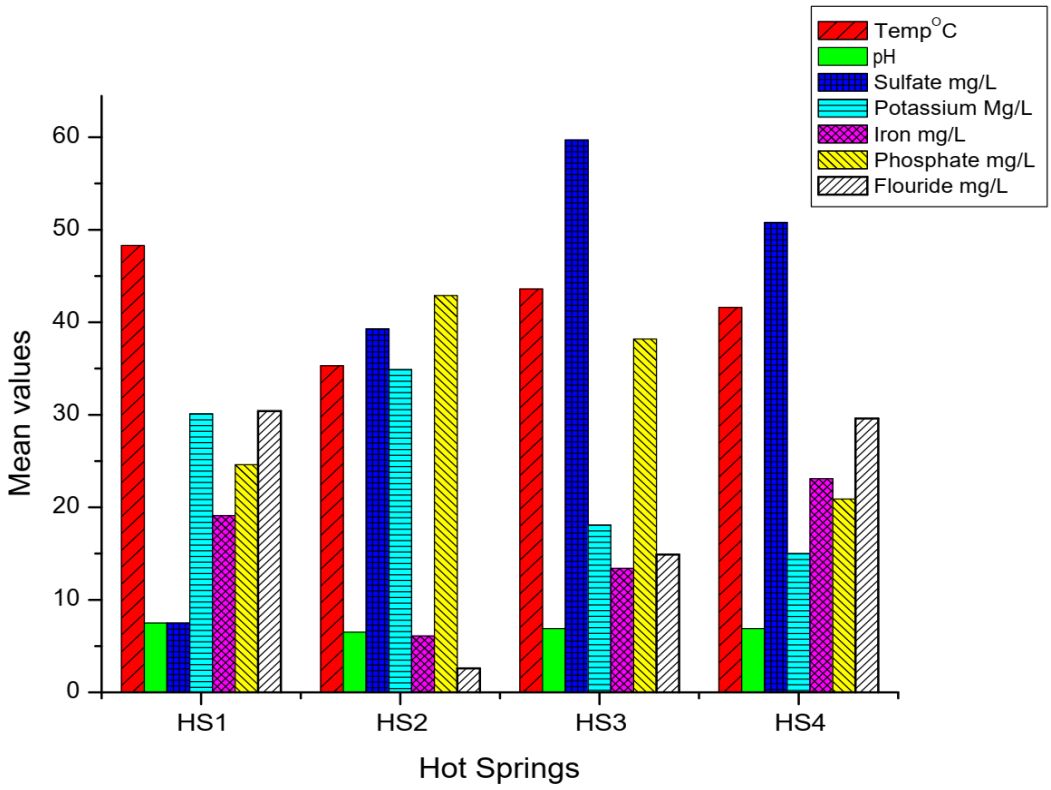


Figure 15 Mean Physicochemical levels of sampled hot springs in Ethiopia, 2018

4.1.2 Microbial Quality of Hot Spring Water Samples

4.1.2.1 *Legionella spp.*

A total of 46 samples out of 106 (43.3%) tested were positive for *Legionella*. Among these 29(27.4%) were from water samples and the remaining 17(16%) from biofilm samples in water distribution system as shown in Table 9.

Table 9 *Legionella* prevalence from sampled hot springs in Ethiopia 2018

	Water Sample tested for *Legionella Spp*						Biofilm Samples		Total Positive samples
	Positive (>10CFU/L)					Negative N (%)	**Positive N (%)**	Negative N (%)	N (%)
Sample source*	10-99 N (%)	100-999 N (%)	1000-9999 N (%)	>10,000 N (%)	**Total +Ve N (%)**				
HS1 N=32	-	1(3.1)	1(3.1)	-	**2(6.25)**	13(40.6)	**8(25)**	9(28.1)	**10 (31.3)**
HS2 N=26	-	3(11.5)	6(23.1)	2(7.7)	**11(42.3)**	12(46.2)	**2(7.7)**	1(3.8)	**13 (50)**
HS3 N=23	-	3(13.0)	2(8.75)	-	**5(21.75)**	6(26.1)	**6(26.1)**	6(26.1)	**11 (47.8)**
HS4 N=25	2(8)	4(16)	5(20)	-	**11(44)**	8(32.2)	**1(4)**	5(20)	**12 (48)**
Total N=106	2(1.9)	11(10.4)	14(13.2)	2(1.9)	**29(27.4)**	39(36.8)	**17(16.0)**	21(19.8)	**46 (43.4)**

**HS1, Hot spring 1; HS2, hot spring 2; HS3, Hot spring 3; HS4, Hot spring 4;CFU,Colony Forming Units*

4.1.2. 2 *Heterotrophic Plate Count*

HPC count from hot spring samples indicated that 23 (21.7%) water samples had *HPC* count from 300 to 400 CFU/mL while the remaining 83(78.3%) samples had *HPC* less than 300 CFU/mL as presented in Table 10.

Table 10 Interval values of *HPC* level from four hot springs in Ethiopia 2018

Sample Source*	<10CFU/mL	10-99CFU/mL	100-299 CFU/mL	300-400 CFU/mL
HS1 (n=32)	13(40.6%)	8(25.0%)	7(21.9%)	**4(12.55%)**
HS2 (n=26)	1(3.8%)	2(7.7%)	13(50%)	**10(38.5%)**
HS3 (n=23)	6(26.1%)	8(34.8%)	7(30.4%)	**2(8.7%)**
HS4 (n=25)	2(8%)	7(28%)	9(36%)	**7(28%)**
Total (106)	22(20.7%)	25(23.6%)	36(34%)	**23(21.7%)**

**HS1: Hot spring 1, HS2: hot spring 2, HS3: Hot spring 3, HS4: Hot spring 4*

The mean Log_{10} expression for *Legionella* ranged from 2.60 (HS3) to 3.22 (HS2) whereas, the mean Log_{10} *HPC* was between 2.42(HS3) to 2.55(HS4). The mean, interval values of Log_{10} expression of *Legionella* positive samples with the *HPC* of sampled hot springs presented in Table 11.

Table 11 Mean and Max Log_{10} Expression of *Legionella* and *HPC* from Hot Springs

source*	Log_{10}CFU/L samples for *Legionella*			Log_{10} CFU/mL samples for *HPC*		
	Mean ±SD	Min.	Max.	Mean ±SD	Min.	Max.
HS1	2.62(0.71)	1.69	3.56	2.44(0.093)	2.34	2.59
HS2	3.22(0.78)	2.00	4.04	2.48(0.062)	2.35	2.57
HS3	2.60(0.69)	1.69	3.77	2.42(0.117)	2.31	2.59
HS4	2.68(0.76)	1.69	3.74	2.55(0.088)	2.47	2.90

**HS1: Hot spring 1, HS2: hot spring 2, HS3: Hot spring 3, HS4: Hot spring 4; Max, Maximum; Min, Minimum*

Hot springs comprises consortia of microorganisms which have environmental significance. Higher level of *HPC* per ml of water sample may indicate the hygienic level or condition of the water system. Some of the *HPC* colonies from the hot spring samples presented in Figure 16 as follows.

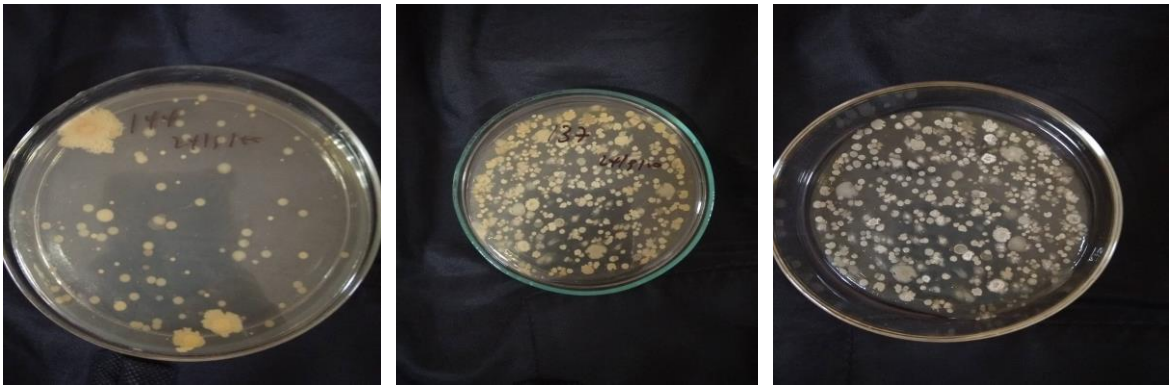


Figure 16 *HPC* colonies from HS CFU/mL, spread plate method, 35°C/48 h plate count agar 2018

Hot springs contain diverse microbial and physicochemical components compared to other natural freshwater bodies (Ohno *et al*., 2003; Matz *et al*., 2003). The detection level of *Legionella* from present study was (43.3%) Table 9, which is higher than the reported percentages from foot spas of Japan 38% by Masato *et al*., (2013), 27.8% by Huang *et al*., (2010), 27% from Japan by Furuhata et *al*., (2004), 23% from Taiwan by Hsu *et al*.,(2006), 21%from Taiwan by Yusen *et al.*,2006) and 22% from Tunisia by (Ghrairi *et al*., 2013). Relatively lower level of *Legionella* detection from hot springs might be resulted due to the good hygiene and sanitation level from sampled hot springs and the physicochemical quality or environment of the hot springs.

However, somewhat higher levels *Legionella* detection than this study findings were reported from elsewhere; as 100% from Poland by, Żbikowska *et al.*,(2013), 93.8% from Taiwan by (Shen *et al*., 2015), 71.9% from Thailand by Sukthana *et al*.,(2005), 51.9% from Beijing by (Qin *et al*., 2013). Higher level of *Legionella* detection in the latter reports could most probably reported due to the physicochemical quality of hot springs, the hygienic or

sanitation level, application of more advanced detection method (like PCR and others) than the traditional culture method used in this study.

Legionella's survival and growth in biofilms of the water distribution system has already been acknowledged by many scientific reports (e.g., WHO, 2011). In this study 16% of the biofilm samples from hot spring water distribution system were positive for *Legionella.* Biofilms are stronghold for survival and dissemination of opportunistic pathogens including *Legionella* and their prevalence indicate the long term persistence in the system (Zineddine *et al*., 2013; Murga *et al.*, 2001).

In the presence of nutrients, scale or corrosion, low flow water and others microbes *Legionella* can form biofilms and this biofilms are an important factor for survival and growth of *Legionella spp* in water system (WHO, 2007). *Legionella* survive and grow in biofilms, sediments and can be ingested by trophozoites of certain free living amoebae which play an important role in their persistence in water environments (WHO, 2011).

Even though disease-causing *Legionella* serogroups was not determined in this study Figure 17, possibility of getting *Legionella* infections from Ethiopian hot springs as aerosols in water vapor cannot be ruled out as the bacteria are expected in closed and semi-closed bathing rooms, which are ideal environments for *Legionella* infection in humans, as it occurs following inhalation of contaminated aerosols generated by the hot water systems (Shih *et al.*, 2010).

Waterborne infections from hot water distribution system devices are a concern for Legionnaires disease outbreaks. In developed countries, *Legionella* are one of the most important water-based bacterial pathogens caused by management failure of engineered water

systems. The colonization of *Legionella* in hot springs or hydrothermal areas may not be surprising due to being warm and lack of treatment in water distribution system. Hence, recreational use of hot springs associated with the use of hot tubs, swimming pools and open waters might be source of *Legionella* infection. The infection risk increases for users of thermal waters, balaneotherapy or healing centers Pond, (2005) such as those Immuno-compromised individuals, diabetics and others visiting hot springs (Kirschner & Alexander 2016).

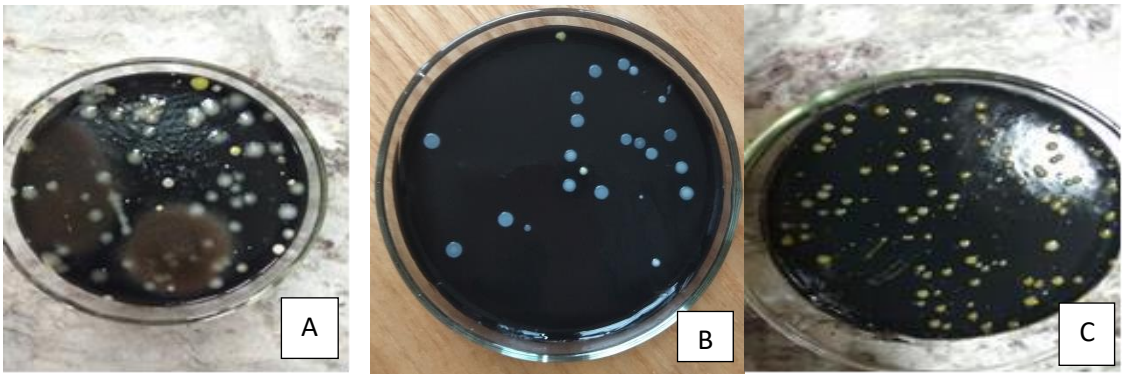


Figure 17 *Legionella spp* with other microbes on BCYE agar (A). *Legionella* positive (B&C) on BCYE agar GVPC supplements; (B) From swab sample, (C) from shower water

The routine testing of microbial water quality parameters like thermotolerant coliforms and *HPC* testing may not indicate the presence or absence of *Legionella* from water distribution system so episodic testing for *Legionella Spp* in water distribution especially from hot tubs is required and management effort should be duly exercised to maintain the operational level of *Legionella Spp* detection <1CFU/100ml (WHO, 2011; WHO, 2006).

Actually *HPC* per mL testing may indicate the hygienic level of water or treatment efficiency specifically the processes of coagulation, filtration and disinfection, on which the microbial populations counts in the system need to be low as possible. Perhaps, *HPC* counts can be

used as an alternative level for monitoring the overall quality of water environment in terms of contamination. Unlike potable water facilities, Hot springs do not have disinfection process that can reduce or eliminate expected microorganism and they may contain higher level of HPC bacteria. Relatively the higher level of HPC might also indicate the presence of stagnant parts of pipe distribution system, availability of nutrients and lack of treatment or residual disinfectants.

Although high *HPC* measurements have not been found to correlate with illness incidence WHO (2003), the *HPC* level does indicate the favorable conditions available for bacterial growth and should be fixed. Depending up on the type and kind of water used for various purposes countries may use different guideline level for *HPC* population per mL that may range from <100CFU/mL to 500CFU/mL (WHO, 2003).

The relationships between the mean Log $_{10}$ *Legionella and HPC* count is presented in Figure 18. An increased level of *HPC* results to certain extent to an increased level of *Legionella* especially to HS2 and HS4 while the level of *LG* is lower concomitant to the lowering of *HPC* for HS1 and HS3. As it has been observed from the correlation from Table 13 all of the sampled hot springs *Legionella* correlated with HPC negatively as HS1(r=-0.528), HS2 (r=-.494), HS3(r=-0.722) and HS4(-0.574). In the present case study the survival of *Legionella* influenced negatively by *HPC.* As Mariam *et al*., (2013) described other microbes might influence the survival of *Legionella spp* in water system negatively or positively.

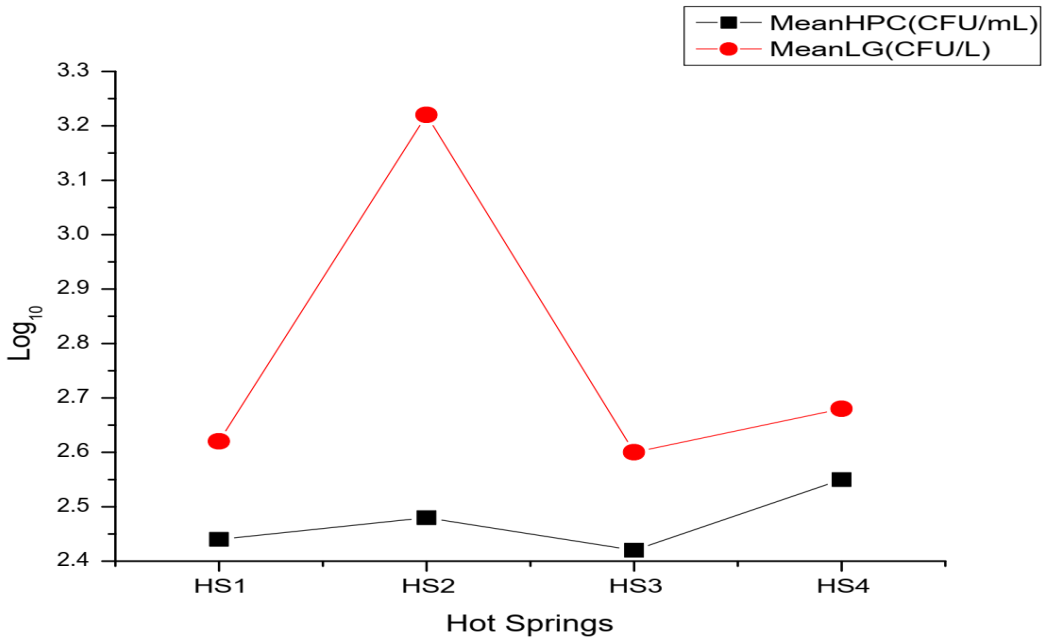


Figure 18 Mean Log_{10} *Legionella* and *HPC comparison* from Sampled Hot Springs

Factors like the available infrastructure and management facilities may contribute for the prevalence of *Legionella* in sampled hot springs. During sampling time it has been observed that all of the sampled hot spring spas produce and distribute hot spring waters through iron based tubes to their shower rooms, bathing room's swimming pools, without using any sort of treatment or use of disinfectants. Only hot spring spa (HS1) uses treated drinking water supplied by the municipality for mixing purpose. The available mean temperature ($48.3^{O}C$), the disinfectant level in treated water together with other factors may contribute to the lower level of CFU for *Legionella* and *HPC* than others Table 9 and10.

The remaining three hot springs (HS2, HS3 and HS4) are using hot spring water directly without mixing with treated drinking water and hence this condition together with others may contribute for the relatively higher level of *Legionella* detection than others. All sampled hot

spring facilities are working in semi closed and closed water distribution system which needs regular management of waterlines from source to point of use. In sampled hot springs the system lacks standardize protocols for to make the water system clean and hygienically safe to the customers this might be one factor for the prevalence of *Legionella*.

So far in Ethiopia, there is no guideline for recreational water management in general and *Legionella* monitoring and risk management schemes in particular. Sampled hot springs water facilities are operating with lack of awareness about emerging pathogens like *Legionella* and others emerging risk or targeted risk management interventions. Excluding hot spring (HS3), all are resorts giving recreation, hotels and modern therapeutic services to customers. All of the hot springs has concrete based reservoirs except hot spring (HS1) which uses both Iron based water tanks (for cold water) are not properly cleaned on regular basis.

Expansion work of buildings and infrastructures is undergoing in all of the hot springs. This condition will result extensive internal water distribution networks (old and new cross connections) and more storage tanks. This may indicate in the possibility of temperature-related microbial growth in water distribution system. Poorly developed and managed water distribution line infrastructures were observed in HS2, HS3 and HS4 than HS1 and this condition may have its role for the observed microbial quality of hot spring water. Most of the sampled hot springs (except HS4) are found within urban area and has been giving services for more than six decades. Hot spring (HS3) is recently developed. Regular customers of the sampled hot springs are tourists (local or foreign), the elderly, Adults, youth and ill people. Especially the elderly and the ill people are immunosuppressed group of the society are susceptible for opportunistic microorganisms infections unless and otherwise proper water safety plan (WSP) management strategy or practices are taken in to consideration.

Hot springs contain diverse physicochemical components than fresh water bodies (Ohno *et al*., 2003 and Matz *et al*., 2003). The mean range Temperature and pH values measured in Ethiopian hot springs were from 35.3-48.3^{O}C and 6.5-7.5 correspondingly. The finding was in agreement within the temperature and pH range described by Diederen, (2008) and Chien, (2006) where the Temperature and pH of sampled hot springs in the range of 0–68^{O}C and 5.0 to 9.0 are considered ideal physical environments for *Legionella Spp* (WHO,2014).

Though *Legionella* was detected from all hot springs, a relatively higher level of *Legionella* was detected from hot spring HS2 than others. This might be due to the observed lower level of temperature and other physicochemical water quality parameters than others. Water temperature is one important aspect that may favor the growth and multiplication of *Legionella.* Lower temperatures supported survival of *Legionella* without loss of cultivability and *Legionella spp* are metabolically active in temperatures of up to 45°C but the cultivability declined with increasing temperature. Relatively water temperature between 25^{O}C to 45^{O}C encourages *Legionella* to grow and multiply in water distribution systems and spa pools WHO, (2007) while *L. pneumophila* can multiply better at temperature of 40^{0}C than others Harm *et al*., (2017) and some scholars also presented *Legionella* can withstand temperature over 60^{O}C (Hilbi *et al.,* 2011).

In this study except HS1 (48.3^{O}C) all others had mean water temperature between >35^{O}C and below 45^{O}C Table 8 indicating the suitability temperature condition for *Legionella* multiplication in sampled hot spring water facilities. Besides enhancing the growth of microorganisms, high water temperature may increase problems related to taste, odor, color, corrosion biofilm formation in water distribution lines that may further contribute to

Legionella growth (WHO, 2011). The association of temperature and percentage of *Legionella* positive samples presented in Figure 19.

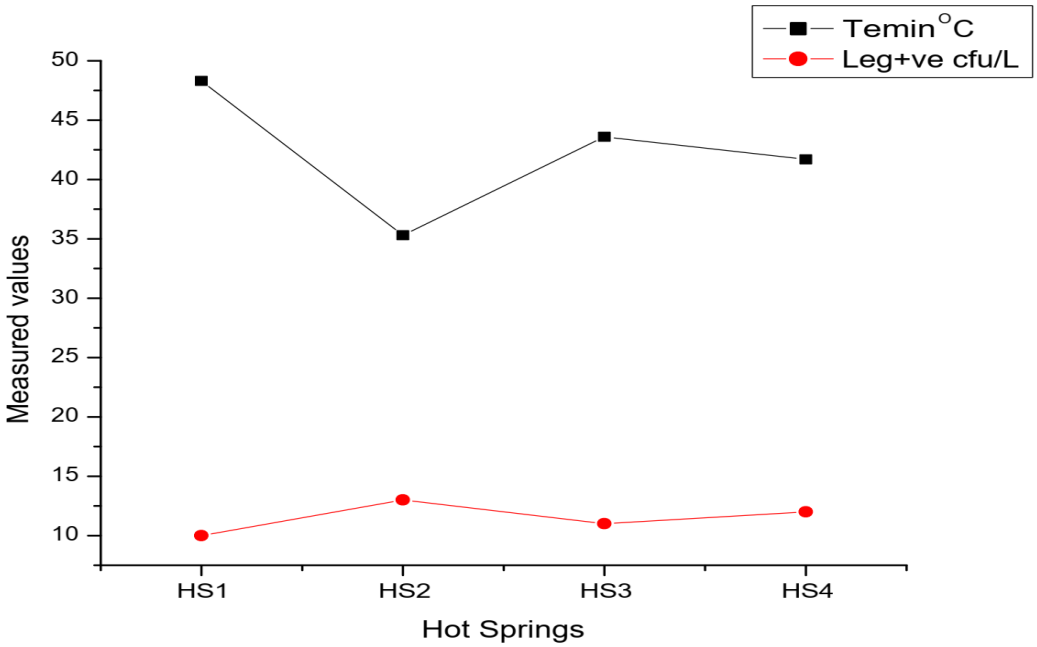


Figure 19 Relation of Temperature with *Legionella* prevalence from sampled hot Springs

Higher number of *Legionella* positive samples were obtained from mean temperature about 35^{O}C (HS2) and from those hot springs that has lower than mean 45^{O}C water temperature (HS3 and HS4). Since *Legionella* can be isolated from hot water temperature between 35 ºC and 45 ºC with the greatest increase in viable counts occurring between 37 ºC and 42 ºC WHO (2007), these findings were in agreement with temperature values reported so far Table 9 and 11 . Besides the other physicochemical parameters, The number of samples from hot springs which have pH closer to 7 were positive for *Legionella* (HS2, HS3 and HS4) than those with pH values closer to 8 (HS1).

Hence, pH and other than others might have its own share for the survival and prevalence of *Legionella Spp* in hot spring water distribution system. Normally, *Legionella Spp* were mostly

isolated within 6-8 pH levels (Kannan *et al*., 2017), hence mean result from our study was in agreement with the mentioned pH range. Moreover, the metabolic activity of *Legionella spp* could be maintained for long periods even in microcosms with pH (6-8) and high concentrations of salt (Ohno *et al*., 2003). The hot spring water pH and their association to *Legionella* prevalence presented in Figure 20.

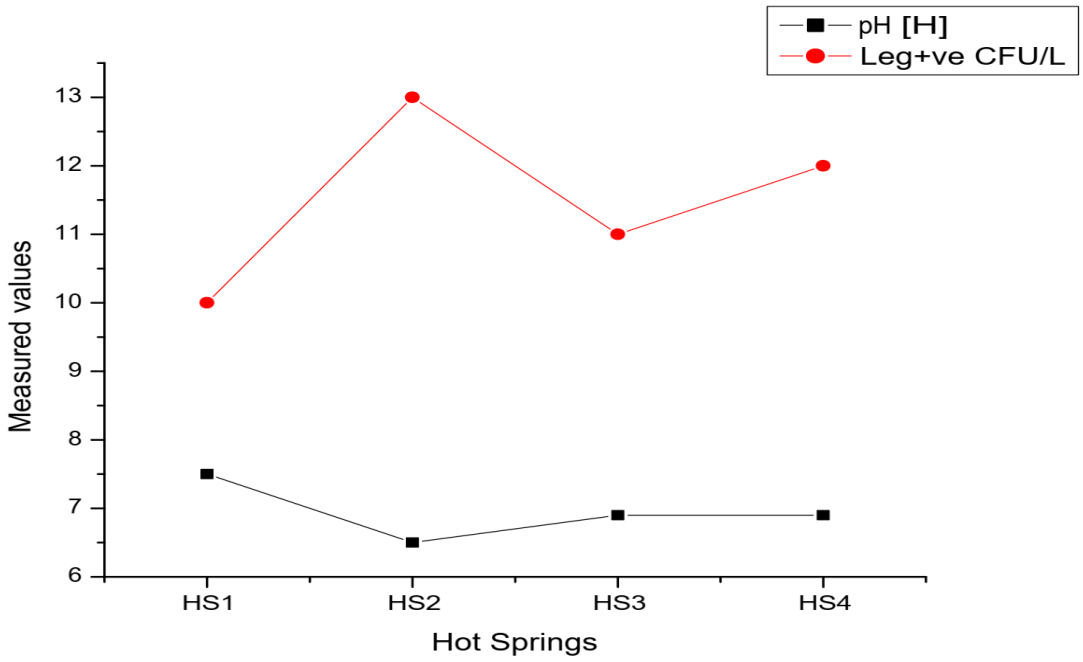


Figure 20 Relation of pH with *Legionella* prevalence from sampled hot Springs

Total dissolved Solids (TDS) Relatively higher level of mean TDS 2569mg/L was measured from hot spring HS3 of this study than reported elsewhere 1608 mg/L by Pet *et al*., (2005), 628 mg/L by Amanuel *et al*., (2017) and 1028.8 mg/L by Memory *et al*., (2015). However relatively higher than this research finding 15552 mg/L mean TDS values reported from Eretria by Amanuel *et al.,* (2017). Moreover the present study reveals that all of the sampled hot springs(HS1- HS4) had higher level of TDS (> 1151.4 mg/L) which is greater

than reported values elsewhere as 628 mg/L ,543 mg/L by Amanuel *et al*,(2017) and 199.4 mg/L and 203.8 mg/L by Memory *et al*., (2015).

TDS less than 600mg/L is good for drinking water but unpalatable when the level greater than 1000 mg/L (WHO, 2011). High level of TDS may result scaling in water pipes, heaters, boilers and appliances and encourage biofilm formation in pipe lines WHO, (2011) and opportunistic pathogens may grow to greater concentration with in the biofilm formed. Hence all of the sampled hot springs water distribution lines are susceptible for scaling, leakages and biofilm development especially HS1 and HS3 which had TDS >2000mg/L Table 12

Comparatively among sampled hot springs in this study, the mean Electrical conductivity (EC) were highest in HS1 (3084.1 mS/cm) and lowest HS2 (1553.1 mS/cm). Some research findings reported as 1153.3mS/cm by Sisay *et al*., (2015), 1317 mS/cm by Amanuel *et al*., (2017). However, as much as 9060 mS/cm and 23133 mS/cm EC from Eretria was reported by Amanuel *et al*., (2017). The mean tested physicochemical parameters of this study compared with other hot springs presented in Table12.

Hot spring 2 (HS2) has got relatively least level of TDS and EC than the other three sampled hot springs the geology and environmental nature of the hot spring that may affect the physicochemical conditions of the hot springs as described earlier. *Legionella* were isolated from all of sampled hot springs having diverse TDS, EC, and alkalinity High value of bicarbonate, sodium, and conductivity was reported from Ethiopian hot springs (Sisay *et al*., 2015a&b).

Iron The mean iron (Fe^{2+}) content of the sampled hot springs was <10 in HS2 (6.1mg/L) and >10 but less than 15 in HS3 (13.4mg/L) while relatively higher in HS1 (19.1mg/L) and HS4 (23.1 mg/L) Table 12. Relatively the lower iron level from sampled Ethiopian hot spring

HS2(6.1mg/L) was much higher than 0.1mg/L reported by Amanuel *et al*, (2017) and 0.3 mg/L by Pet *et al*., (2005). Iron levels ranging from 0.5 to 50 mg/L might be expected from natural fresh waters (WHO, 2011) as the main Iron source might be the geological nature of hot springs or the corrosion resulted from the old iron pipe line based water distribution system (Pet *et al.,* 2005).

Even though it is not correlated, relatively higher level of Iron level together with *Legionella* prevalence reported from all sampled hot springs indicated Iron may not have influence to *Legionella* prevalence in hot springs. But Iron in water distribution system may stains laundry and plumbing fixtures if concentration >0.3mg/L and promotes the growth of "iron bacteria", biofilm formation on piping and such conditions may enhance growth of *Legionella* and other microbes in water distribution system (WHO, 2011).

Fluoride (F^-) the mean fluoride level measured in this study was 2.6 mg/L (HS2), 14.9 mg/L (HS3) 30.4 mg/L (HS1) and 29.6 mg/L (HS4). Except HS2 all of sampled hot springs had relatively higher level of fluoride than the reported values as 8.2 and 6.5 mg/L by Amanuel *et al*., (2017), 6.7 mg/L by Pet *et al*., (2005) and Ranst *et al*., 2007). On other hand, some fluoride level study were relatively higher than measurement of HS2 as 3.2 mg/L by Memory *et al.,* (2015), and 2.9 mg/L by Amanuel *et al*.,(2017). Memory *et al.,* (2015) reported much lower level of fluoride between 1.01 mg/L to 2 .01 mg/L than this finding Table 12.

The source of fluoride may be the presence of fluorite deposit in the hot spring (Pet *et al*., 2005) or concentrations in water can be linked to volcanic activities as the sampling sites are within or near the great Rift Valley region (RVR) of Ethiopia on which the high fluoride level is reported (Jovine *et al*., 2017). In some instances it may be due to the presnsence of alkaline

silicic volcanic rocks like pantelleritic obsidians, as fluorine-bearing mineral fluoride was reported up to 17mg/l in cold ground water and 40mg/L in Shalo hot springs (Vladimír *et al*., 2015).

In the present study report *Legionella* was not strongly correlated with fluoride level of most sampled hot springs except HS4 (r=-0.497) Table 13 but *Legionella* were isolated from all of sampled hot springs that has relatively higher level of fluoride. This may indicate fluoride level may not have such negative influence for survival of *Legionella*. Since people are drinking hot spring water for curing certain illnesses, the high level of fluoride from sampled hot springs may pose additional health problem since WHO do not recommends drinking water containing fluoride >1.5mgL may pose serious health problem to dentine and bone of humans.

Sulfate (SO_4^{2-}) the presence of sulfate in water can cause noticeable taste, and very high levels might cause a laxative effect in unaccustomed consumers. Taste impairment varies with the nature of the associated cation; taste impairment is minimal at levels below 250 mg/L (WHO, 2011). The maximum sulfate level in the present study 59.7 mg/L (HS3) was much lower than the 127.8 mg/L reported by Pet *et al*., (2005). The main source of the sulfate attributed to ground water which has relation to atmospheric, geologic and biological process. As hot springs had ground water in origin highest level of Sulfate may be expected (WHO, 2004 and ESEPA, 2003). Besides the noticable test, ingestion of high level Sulfate >500mg/L may have gastrointestinal effects to certain people and may contribute to the corrosion of distribution systems (WHO,2011).

Alkalinity($CaCO_3^{2-}$) the total mean alkalinity in sampled hot springs was 1001.9 mg/L (HS2), 1797.4 mg/L (HS3), 2072.8 mg/L (HS1) and 4752 mg/L (HS4) Table 12, which

relatively higher than some research reports of hot springs as 27.7mg/L, 341.0 mg/L and 394.3 mg/L respectively by Amanuel *et al.*, (2017), 248.0 mg/L by Pet *et al.*, (2005) and 1264.7 mg/L by Rajapaksha and Maithreepala, (2014) Table 12

The presence of high level of alkalinity in water distribution system may contribute to the development of biofilm though scale formation and these may lead to corrosion, contamination and damage to water pipes tanks and appliances (WHO, 2011). High level of alkalinity in water might be associated to the chemical reaction of water system while thermal water flows through wall rocks that may contain limestone, dolometic-limestone and dolomite (Amanuel *et al*., 2017; Rajapaksha and Maithreepala, 2014; Pet *et al.*, 2005). The alkalinity levels found in this study may have resulted problems related to corrosion, scale and biofilm formation and this conditions may favor the growth and multiplication of opportunistic pathogens including *Legionella spp* and others.

Hot springs are preferred by most people because of the diverse physicochemical conditions that hot springs contain which might be suitable for bathing, recreation, health or therapy purposes (Zaini *et al.*, 2013 and Yaowalark *et al*., 2005). Mostly, the solubility of minerals increases in hot water. Hence thermal springs usually have concentrated trace elements. The chemical composition of spring water depends largely on the composition of the rain water, its temperature and pH, and the geology of the aquifer and the rocks through which the water rises to the surface (Olivier *et al*., 2008).

However the physicochemical and microbial quality of hot springs may favorable conditions for growth and multiplication of some opportunistic pathogens within the existed water distribution system. Hence, safe water management plan and close follow-ups are required.

Table 12 Mean ±SD physicochemical parameters of sampled hot springs compared with literatures [a]

Locations*	Temperature	pH	TDS Total Dissolved Solids	EC Electrical Conductivity	SO_4^{2-} Sulfate	K^+ Potassium	Fe^{2+} Iron	PO_4^{2-} Phosphate	F^- Fluoride	$CaCO_3^{2-}$ Alkalinity	References
ETH	48.3 ±5.4	7.5 ±0.3	2433.4 ±192.7	3084.1 ±369.6	7.5 ±3.6	30.1 ±15.1	19.1 ±7.1	24.6 ±11.6	30.4 ±7.8	2072.8 ±244.1	1
	35.3 ±1.4	6.5 ±0.2	1151.4 ±16.5	1553.1 ±79.8	39.3 ±4.9	34.9 ±5.6	6.1 ±2.3	42.9 ±12.3	2.6 ±0.7	1001.9 ±85.4	
	43.6 ±3.3	6.9 ±0.2	2569.4 ±286.4	2937.4 ±112.8	59.7 ±14.5	18.1 ±1.4	13.4 ±1.1	38.2 ±12.1	14.9 ±1.1	1797.4 ±285.1	
	41.6 ±3.9	6.9 ±.3	1463.4 ±103.1	2012.5 ±168.3	50.8 ±14.5	15.0 ±2	23.1 ±11.9	20.9 ±3.3	29.6 ±7.3	4752.0 ±665.3	
ETH	43.5 8±2	7.9 ± 0.5	-	1153.3 ±629.7	-	-	-	0.18 ±0.2	-	-	2
ERI	49.2 ±0.9	7.22 ±0.0	628 ±26	9060 ±116	44.0 ±3.5	104 ±3	-	-	8.20 ±1.3	341.0 ±9.5	3
	51.3 ±0.6	7.30 ±0.0	15552 ±585	23133 ±696	272.7 ±12.7	198 ±7	-	-	2.87± 0.40	27.7 ±0.7	
	51.4 ±0.5	8.05 ±0.0	543 ±38	1317 ±117	75.3 ±1.2	13 ±2	0.10 ±0.01	-	6.48 ±1.1	394.3 ±9.8	
SAF	43	8.19	199.36	44.0	9.26	1.14	-	0.0	3.16	-	4
	45	9.24	203.76	39.0	18.20	1.1	-	0.0	1.01	-	
	43.9	7.20	-	273.0	170.0	15.0	-	-	2.00	-	
	41	7.63	1028.8	330.0	143.6	21.8	-	24.9	2.24	-	
THA	58 ±17	7.6 ±7.9	1608 ±3873	-	127.8 ±286	18 ±38.7	0.3 ±0.4	-	6.7 ±6	248. ±116	5
SLA	44.1 ±0.6	7.52 ±0.3	-	9.62 ±3.3	-	-	-	0.07± 0.2		1264.7 ±30.5	6

[a] *Data reported in mg/L except for pH, EC (mS/cm) and Temperature (^OC): *ETH, Ethiopia; ERI, Eritrea; SAF, South Africa; THA , Thailand; SLA, Sri Lanka ; 1=this study, 2=Sisay et al,(2015) ,3=Amanuel et al.,(2017), 4=Tekere et al.,(2015),5=Pet et al., (2005), 6= Rajapaksha, (2014)*

4.1.3 Associations of Microbiological Parameters and Abiotic Factors

In the investigated hot spring water samples most of the microbial and abiotic factors are not strongly correlated with the tested parameters. But the correlation matrix among the tested physicochemical parameters with microbial parameters revealed that, *Legionella* was not significantly correlated with most tested physicochemical parameters except two TDS and sulfate (HS1), Temperature and Phosphate (HS2) Fluoride (HS4) which were all weakly correlated. Positive correlation at $P<0.01$ level was observed between *HPC* with TDS($r=0.463$), *HPC* with Sulfate ($r=0.600$) in HS1 on the other hand $P<0.05$ level was observed between Temperature of *HPC* with Phosphate ($r=0.454$) in HS2: *Legionella* with Temperature ($r=0.424$) in HS2; *Legionella* correlated negatively with Fluoride ($r=-0.497$) in HS4. Negative correlation between *Legionella* and *HPC* was observed from all samples at $P<0.01$ from HS1($r=-0.528$), HS3($r=-0.722$), HS4 ($r=-0.574$) While *Legionella* was negatively correlate with *HPC* from HS2 at $P<0.05$ ($r=-0.494$) Table13 and 14.

Table 13 Pearson Correlations of the Microbial, physicochemical parameters of Hot Springs, 2018*

	Temp	pH	TDS	EC	SO_4^{2-}	K^+	Fe^{2+}	PO_4^{2-}	F^-	$CaCO_3$ $_{2-}$	HPC	LEG
HPC[a]	-.020	.271	**.463****	.133	**.600****	.018	-.047	.306	.038	-.030	1	
HPC[b]	-.103	.048	-.003	.117	.073	-.025	.017	***.454****	.182	-.094	1	
HPC[c]	-.309	-.142	-.232	-.204	.129	.265	.013	.261	.163	-.296	1	
HPC[d]	-.379	-.218	.231	.078	.158	-.033	-.095	.079	.347	.159	1	
LEG[a]	-.224	.139	-.117	.012	-.289	-.297	.053	.072	.085	-.240	**-.528****	1
LEG[b]	***.424****	-.223	.233	.040	.016	.001	-.069	-.091	.136	-.160	***-.494****	1
LEG[c]	-.057	.132	-.327	-.363	-.149	-.144	-.012	-.239	-.324	.049	**-.722****	1
LEG[d]	.096	.320	-.300	-.015	-.117	.117	.171	-.122	***-.497****	-.238	**-.574****	1

NB: *[a]=HS1; [b]=HS2; [c]=HS3; [d]=HS4; Temp=Temperature; pH; TDS=Total Dissolved Solids; EC=Electrical Conductivity;* SO_4^{2-}*= Sulfate;* K^+*= Potassium;* Fe^{2+}*= Iron;* PO_4^{2-}*= Phosphate;* F^-*= Fluoride;* $CaCO_3^{2-}$*=Alkalinity; HPC=Heterotrophic Plate Count; LEG= Legionella. Boldface; * Correlation is significant at the 0.05 level, **. Correlation is significant at the 0.01 level (2-tailed).*

Nonlinear form of correlation observed with in most of the physicochemical tested parameters in hot springs but some significantly correlated in both directions. The Correlations of within the physicochemical tested parameters of four Hot springs presented in Table 14.

Table 14 Pearson Correlations of the physicochemical quality of Hot springs Ethiopia, 2018 [a]

Sample site	Parameters	Temp	pH	TDS	EC	SO_4^{2-}	K^+	Fe^{2+}	PO_4^{2-}	F^-	$CaCO_3^{2-}$
HS1	SO_4^{2-}	0.048	0.174	**.575****	-0.019	1					
HS2	SO_4^{2-}	0.109	0.089	0.309	-0.099						
HS3	SO_4^{2-}	0.063	-0.129	0.186	0.144						
HS4	SO_4^{2-}	0.298	0.117	-0.122	0.247						
HS1	K^+	-0.151	-0.195	0.063	0.193	0.238	1				
HS2	K^+	0.174	-0.263	**-.620****	0.013	-0.135					
HS3	K^+	-0.282	-0.3	-0.024	0.029	0.126					
HS4	K^+	0.044	0.387	0.238	0.261	-0.104					
HS1	Fe^{2+}	0.34	0.331	0.188	0.05	0.155	**-.385***	1			
HS2	Fe^{2+}	-0.052	-0.15	-0.19	0.146	0.036	-0.04				
HS3	Fe^{2+}	0.222	**-.491***	0.217	0.229	**.642****	-0.021				
HS4	Fe^{2+}	-0.014	0.353	-0.116	-0.107	0.342	-0.04				
HS1	PO_4^{2-}	-0.122	**.455****	**.416***	0.211	**.430***	**.393***	0.06	1		
HS2	PO_4^{2-}	-0.051	0.117	0.181	0.193	0.233	-0.196	0.282			
HS3	PO_4^{2-}	0.344	-0.084	0.27	0.178	-0.004	0.057	0.021			
HS4	PO_4^{2-}	-0.394	-0.343	0.04	0.345	-0.073	0.129	-0.329			
HS1	F^-	0.174	0.021	0.189	-0.123	0.037	-0.273	-0.238	0.044	1	
HS2	F^-	0.19	-0.11	-0.222	0.06	**.510****	0.368	0.051	0.095		
HS3	F^-	-0.036	-0.335	0.228	0.338	0.241	0.032	0.379	-0.14		
HS4	F^-	**-.400***	**-.686****	0.251	0.221	0.06	-0.253	**-.471***	**.583****		
HS1	$CaCO_3^{2-}$	-0.192	**-.369***	-0.288	-0.047	-0.021	**.405***	**-.352***	0.034	**-.377***	1
HS2	$CaCO_3^{2-}$	-0.141	0.087	-0.137	0.303	-0.101	0.138	0.269	0.221	-0.248	
HS3	$CaCO_3^{2-}$	0.002	**.425***	0.074	-0.029	-0.166	-0.137	-0.389	-0.113	-0.044	
HS4	$CaCO_3^{2-}$	0.389	-0.059	0.18	0.22	**.479***	0	-0.075	-0.081	0.078	

NB [a] *Temp=Temperature; pH; TDS=Total Dissolved Solids; EC=Electrical Conductivity;* SO_4^{2-}*= Sulfate;* K^+*= Potassium;* Fe^{2+}*= Iron;* PO_4^{2-}*= Phosphate;* F^-*= Fluoride;* $CaCO_3^{2-}$*=Alkalinity* ***Boldface****,*. Correlation is significant at the 0.05 level, **. Correlation is significant at the 0.01 level (2-tailed).*

Legionella occurrence and infection from hot spring resorts, spa, hot tubs, swimming pool Chien *et al*., (2006), Tian *et al*., (2013) and in some hot springs as infection hot spot (Shu *et al*., 2015) and source of *Legionella* infection in Japan (Masato *et al*., 2013) reported. Likewise, the prevalence of *Legionella* and other pathogenic free living amoebae (*Naegleria* and *Acanthamoeba)* reported by Yaowalark *et al*.,(2005) from Thailand hot spring and Hsu *et al*., (2010) from Taiwan spring recreation areas. These and other research findings indicating hot springs may harbor opportunistic pathogens; can be considered as potential source of microbial infection (Jocelyn *et al*., 2017).

In fact the possible source of *Legionella* infection might be associated with a number of comorbidity factors including physicochemical water quality parameters (e. g. Temperature), health and susceptibility of the population utilizing the water resources and environmental hygiene and sanitations conditions of the facilities. Like in other developing countries, the environmental hygiene and sanitation conditions around hot springs and recreational areas are ill defined and proper attention is not given due to lack of awareness or willingness to take proper action.

It has been observed during our field visits, the environmental hygiene and sanitation conditions of hot springs were poorly managed and these conditions may contribute for the prevalence of *Legionella* from sampled hot springs. Unless proper water management safety is in place, there might be the possibility of infection from *Legionella* and other opportunistic pathogens while visiting hot spring facilities (WHO, 2011).

4.2 Hospital Water Distribution System

A total of 57 water samples were collected from the two hospitals. Among these 34 water samples were collected from Zewditu hospital (ZH). Specifically, 24 (70.6%) of samples were cold water and the remaining 10(29.6%) were hot water samples. From Hospital water distribution system 4(11.8%) samples were taken from shower tap, 10 (29.6%) from tap water and 20 (58.8%) from biofilm samples.

With respect to Yekatit 12 Hospital (Y12H), a total of 23 water samples were collected. Among these 6(26.11%) were from shower tap, 8(34.8%) from tap, 2(8.7%) from storage tank, 7(30.4%) were from biofilm samples. Generally 12(52.2%) water samples taken from hot water distribution system while the remaining 11(47.8%) from cold water distribution system. The sampling sources of the two hospitals presented in Figure 21.

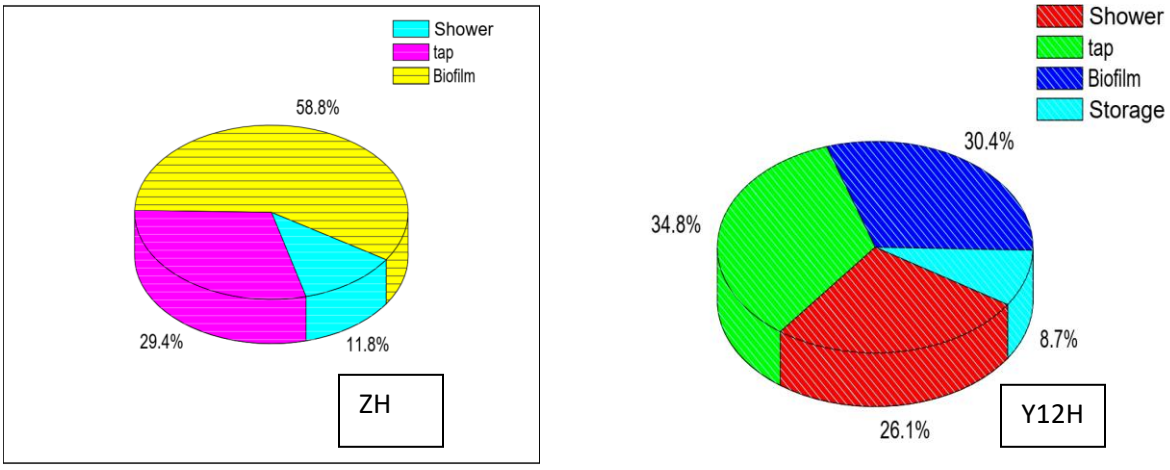


Figure 21 Sampling points from Zewditu and Yekatit12 Hospitals Addis Ababa, 2018

4.2.1 Physicochemical Water Quality of Hospitals Distribution System

The mean Temperature, pH, TDS and EC from Zewditu and Yekatit 12 Hospitals were 27.1^{o}C, 7.4, 88.7mg/L, 130.4 (µS/cm) and 29.3^{o}C, 6.9, 130 mg/L, 194.5 (µS/cm) respectively. Most of the physicochemical water quality measurements were within the guideline values of WHO for drinking water but except the mean Iron level at Yekatit 12 hospital (Y12H) (0.57mg/L) and the available disinfectant level in water distribution system which was 0.16mg/L and 0.19mg/L from Zewditu hospital (ZH) and Y12H respectively. The mean and guideline values of the tested physicochemical parameters from two hospital water samples are presented in Table 15.

Table 15 Mean and range values of physicochemical parameters from Hospital WDS [a] 2018

Parameters	ZH(N=34)		Y12H(N=23)		WHO**	CES *# (MPL)
	Mean ±SD	Range	Mean ± SD	Range		
Temp.	**27.1±9.9**	18.4-51.2	**29.30±12.2**	15.3-44.3		-
pH	7.4±0.3	6.7-8.16	6.9±0.4	6.2-7.5	6.5–8.5	6.5-8.5
TDS	88.7±14.2	68.6-124.6	130.4±23.4	87.3-151.9	1000	1000
EC	130.4±21.9	94.9-177.6	194.5±32.7	134.0-228.0	400-1200	-
SO_4^{-2}	3.75±2.4	0.0-8.0	4.50±2.7	0.0-10.0	500	250
K^+	**1.90±0.43**	1.2-2.8	**2.24±0.34**	1.7-2.7	50	**1.5**
Fe^{2+}	**0.34±0.25**	0.03-0.80	**0.57±0.29**	0.05-1.2	**0.3**	**0.3**
PO_4^{2-}	5.36±3.60	3-17.40	5.58±2.66	2.60-11.3	-	-
F^-	0.42±0.61	0.19-3.50	0.21±0.17	0.03-0.44	1.5	1.5
$CaCO_3^{2-}$	68.97±46.8	25-310	47.17±11.95	35.0-75.0	600	200
Free Cl_2	**0.16±0.3**	0.0-1.00	**0.19±0.3**	0.0-0.8	0.2 - 0.5	0.5

[a] Data reported in mg/L except for pH, EC (µS/cm) and Temperature (^{O}C); MIN, Minimum; MAX, Maximum; ZH, Zewditu Hospital; Y12H,Yekatit 12 Hospital; [**] WHO standards for drinking water

*quality; *# CES, Compulsory Ethiopian Standard drinking water Specifications; MPL, Maximum Permissible Level*

Water Temperature of Yekatit 12 hospital was relatively higher than the Zewditu hospital water samples because hot water was more available at Y12H than ZH. Similarly, higher mean values (mg/L) of Sulfate, Potassium, Iron, Phosphate and lower level of Fluoride were observed from water samples of Yekatit 12 Hospital than Zweditu hospital Figure 22.

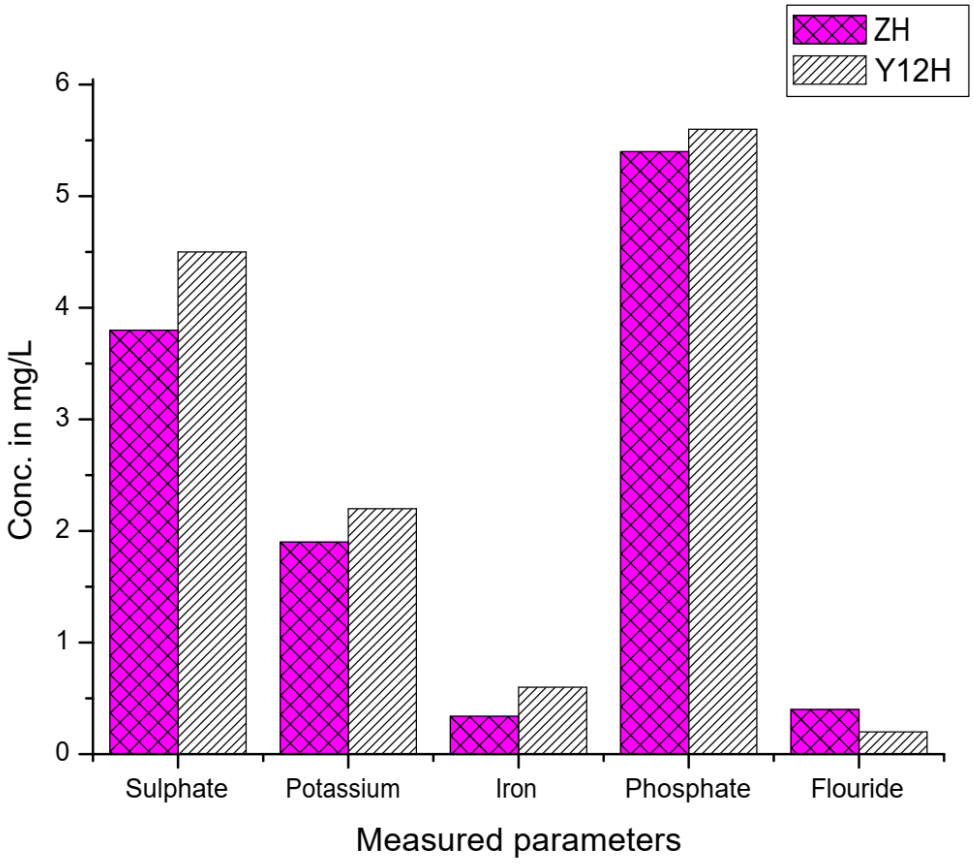


Figure 22 Mean physicochemical water quality values from Hospitals WDS

The mean concentrations of physicochemical water parameters from two hospitals in Addis Ababa presented in Figure 23. Electrical conductivity and total dissolved solids were relatively higher at Y12 hospital than ZH.

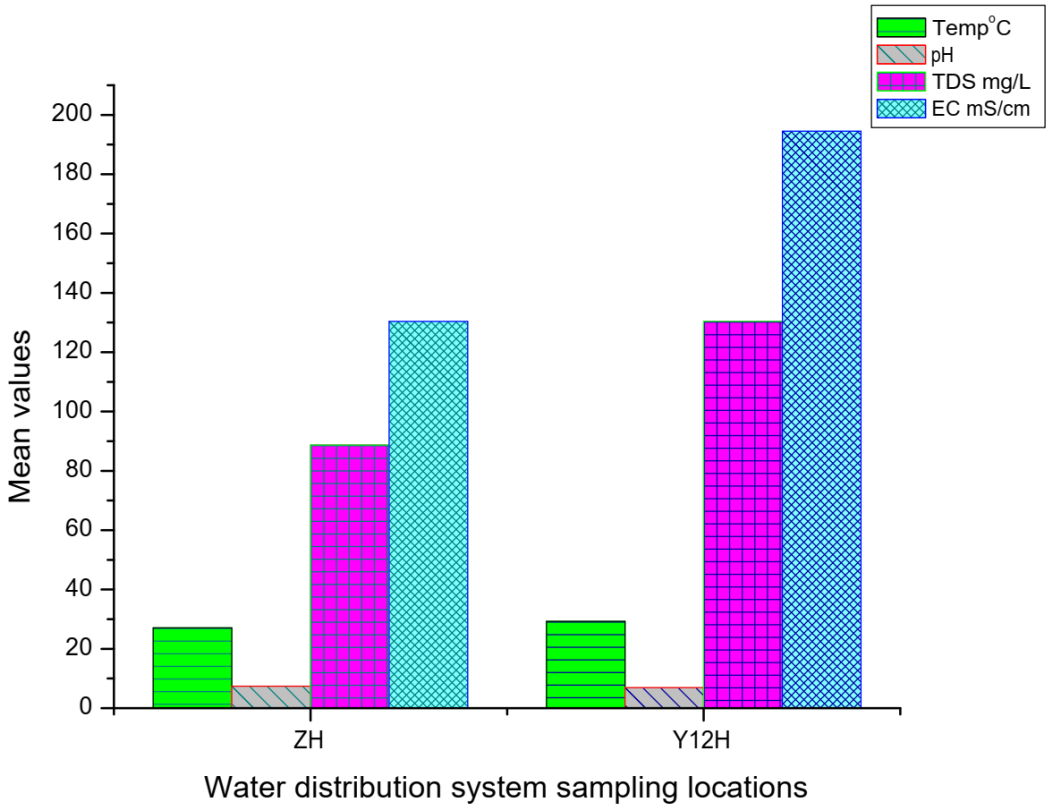


Figure 23 Mean level of water temperature, pH, TDS and EC from ZH and Y12H

4.2.2 *Legionella Spp* and *HPC* from Hospital Water Distribution System

4.2.2.1 *Legionella Spp*

Among tested 57 water and biofilm samples 12/34 (35.3%) from ZH and 10/23(43.5%) from Y12 hospital water samples were positive for *Legionella spp*. Moreover, 9/34(26.5%) biofilm samples from ZH has been positive for *Legionella Spp* indicating *Legionella* dominance from biofilm in water distribution line than water samples. On the contrary most of water

samples from Y12H 10/23 (43.5%) were positive for *Legionella* and all of the biofilm samples from Y12H water distribution system were negative.

Only 7/23(30.4%) water samples from Y12H had *Legionella* levels >1000CFU/L and less than 1000CFU/L of *Legionella* was counted from 3/34(8.8%) samples from ZH and 3/23(13.04%) from Y12H. Moreover, among tested hospital distribution system water samples 5/34 (14.7%) from ZH and 14/23 (60.9%) from Y12H had HPC > 200CFU/mL Table 16.

Table 16 Level of *Legionella* and *HPC* from selected Hospital water distribution system

	Water Sample tested for *Legionella Spp* CFU/L						Biofilm Sample		**Total +ve N (%)**
Source	Positive (>10CFU/L)					Negative N (%)	**Positive N (%)**	Negative N (%)	
	10-99	100-999	1000-9999	>10,000	**Total N (%)**				
ZH (N=34)	1(2.9%)	2(5.9%)	-	-	**3(8.8)**	11(32.4)	**9(26.5)**	11(32.4)	**12(35.3)**
Y12 (N=23)	-	3(13.0%)	4(17.4%)	3(13.0%)	**10(43.4)**	6(26.1)	**ND***	7(30.4)	**10 (43.4)**
	Water samples tested for *HPC* CFU/mL								
Source	<10	10-99	100-199	200-299	300-399	400-499 CFU/mL		Missing	
ZH (n=34)	19(55.9%)	4(11.8%)	6(17.6%)	**4(11.8%)**	**1(2.95)**	-		-	
Y12H (n=23)	-	-	2(8.7%)	**3(13.05)**	**7(30.4%)**	**4(17.4%)**		7(30.4%)	

NB: ZH=Zewditu Hospital; Y12= Yekatit 12 Hospital medical College; ND=note detected*

4.2.2.2 Heterotrophic Plate Count/ *HPC*

The mean ±SD, Minimum and Maximum colony counts of *Legionella* and *HPC* and their Log_{10} expression is presented in Table 17 and Figure 24. The Mean *HPC* colonies from ZH were recorded as 2.2 CFU/mL while 2.4 CFU/mL from Y12H. Maximum *Legionella spp* was detected from Y12H as 4.15 (CFU/L).

Table 17 Mean ±SD of *Legionella* CFU/L and *HPC* CFU/mL & Log_{10} expression hospitals WDS

Microbial parameters	Y12H			ZH		
	Mean ± SD	Minimum	Maximum	Mean ± SD	Minimum	Maximum
Log_{10}*HPC* CFU/mL	2.47±0.16	2.04	2.62	2.24±0.15	2.04	2.55
Log_{10} LGW CFU/L	2.8±1.41	1.0	4.15	1.22±0.46	1.0	2.39

NB: LGW, Legionella from Water; Y12H, Yekatit 12 Hospital; ZH, Zewditu Hospital; CFU, Colony Forming Units

The detection level of free residual chlorine, *Legionella and HPC* from hospital water distribution system is shown in Figure 24 that close association were observed between *HPC and Legionella* in the water samples of Y12 Hospital than the Zewditu Hospital. Meanwhile available mean free residual chlorine from both hospitals water samples was below the required level (<0.2mg/L).

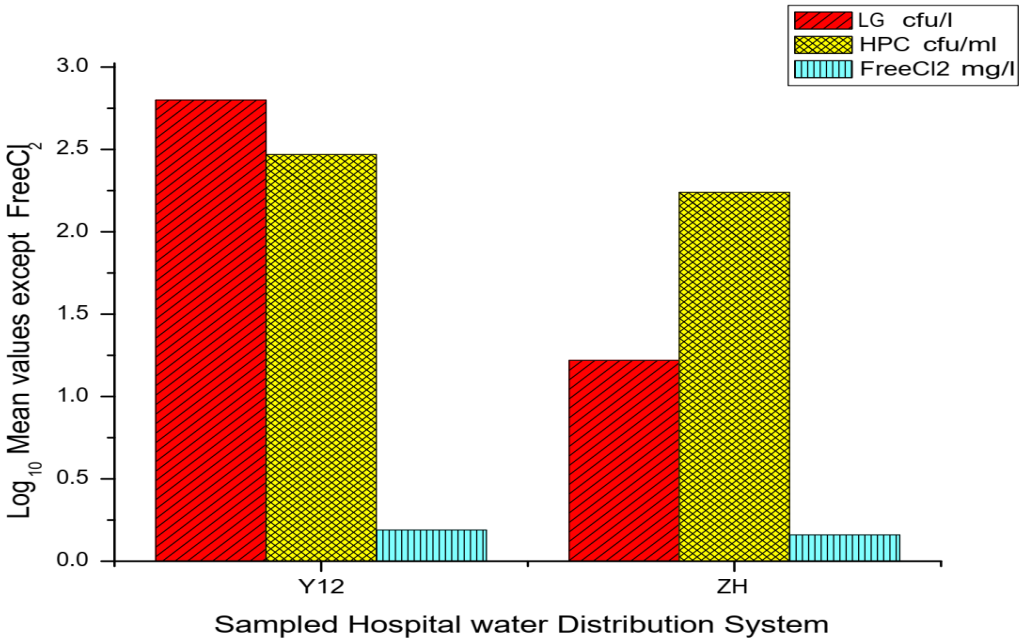


Figure 24 Mean Log_{10} *HPC, Legionella* and residual chlorine (mg/l) levels from hospital samples

4.2.3 Associations of Microbiological and Abiotic Factors from Hospital Water Samples

Associations of microbiological parameters and abiotic factors from sampled two hospitals water distribution systems are presented on Tables 18 and 19. The microbial and abiotic water quality parameters tested from Zewditu hospital are correlated with each other with certain level. From the correlation matrix Table 18, the tested physicochemical and microbial parameters revealed positive and negative correlations at $p<0.01$ and $P<0.05$ significance level.

Water samples from ZH had positive correlations at $p <0.01$ were seen between microbial parameters LGW and pH ($r = 0.456$), LGW and EC ($r = 0.467$) and between physical parameters EC and Temperature ($r = 0.709$), EC and pH ($r = 0.626$), EC and TDS ($r = 0.805$),

TDS and Temperature(r = 0.592). On contrary, negative correlations were observed at P<0.01 between Free Residual Chlorine and *HPC* (r = -0.890), Free Residual Chlorine and LGW (r = -0.459), *HPC* and LGW (r = -0.461).

At p<0.05 level of significance weak negative correlations were observed between Free Residual Chlorine and Temperature (r = -0.383), Free Residual Chlorine and pH (r=-0.345), Free Residual Chlorine and TDS (r = -0.397), Free Residual Chlorine and EC (r=-0.406). Similarly, EC with *HPC* (r=-0.372), EC with PO^{2-}_4 (r=-0.379), EC and $CaCO^{2-}_3$ (r = -0.347) while positively correlated associations seen between Temperature and LGW(r = 0.343), Temperature and pH (r = 0.372), TDS and pH (r= 0.34) at P<0.05 level Table 18

Table 18 Spearman's rho Correlation of physicochemical and microbial parameters from ZH, 2018

ZH	**T^OC**	**pH**	**TDS**	**EC**	**SO_4^{2-}**	**K^+**	**Fe^{2+}**	**PO_4^{2-}**	**F^-**	**$CaCO_3^{2-}$**	**FCl_2**	**LGW**	**HPC**
T^OC	1												
pH	**.372***	1											
TDS	**.592****	**.342***	1										
EC	**.709****	**.626****	**.805****	1									
SO_4^{2-}	-0.072	0.023	***.398****	0.297	1								
K^+	0.287	-0.241	0.196	0.045	0.192	1							
Fe^{2+}	0.227	-0.238	-0.016	-0.112	0.051	**.724****	1						
PO_4^{2-}	-0.19	-0.111	-0.179	**-.379***	0.138	0.007	0.244	1					
F^-	0.202	0.174	-0.054	0.064	**-.489****	0.002	-0.144	0.06	1				
$CaCO_3^{2-}$	-0.114	**-.453****	-0.17	**-.347***	0.071	**.413***	**.400***	**.343***	-0.255	1			
FCl_2	**-.383***	**-.345***	**-.397***	**-.406***	0.063	-0.175	-0.126	0.015	-0.331	0.255	1		
LGW	**.343***	**.456****	**.397***	**.467****	0.18	0.193	-0.127	-0.155	0.299	-0.249	**-.459****	1	
HPC	-0.269	-0.309	-0.264	**-.372***	-0.03	-0.181	-0.123	0.14	-0.163	0.156	**-.890****	**-.461****	1

*NB *. Correlation is significant at the 0.05 level; **. Correlation is significant at the 0.01 level. LGW, Legionella from water; HPC, Heterotrophic plate count; FCl_2, Free residual Chlorine;* $CaCO_3^{2-}$, Alkalinity

Correspondingly, from Yekatit12 hospital water samples associations at P<0.01 and P<0.05 presented in Table 19. Positive correlations at p<0.01 level was observed between Temperature and pH (r = 0.886), Temperature and Sulfate (r = 0.528), Temperature and Potassium(r = 0.662), EC and TDS (r = 0.865), EC and K^+ (r = 0.586), K^+ and TDS (r = 0.717). With the same level of P<0.01, significant negatively correlated associations were observed between Free Residual Chlorine and *Legionella* (r= -0.751), *HPC* and Temperature (r= -0.570), *HPC* and pH (r= -0.584), K^+ and pH (r= -0.646), F^- and TDS (r= -0.539).

At P<0.05 significant level few negative associations were observed between Temperature and LGW (r=-0.416), Free Residual Chlorine and *HPC* (r=-0.502), $CaCO_3^{2-}$ and pH (r= -0.530) and TDS with Temperature (r= -0.520). On the contrary some parameters were positively associated include LGW and *HPC* (r= 0.464), F^- with pH (r= 0.427), Fe^{2+} and TDS (r= 0.509) Table 19.

Table 19 Spearman's rho Correlation of physicochemical and microbial parameters from Y12H

Y12	T ^{O}C	pH	TDS	EC	LGW	HPC	SO_4^{2-}	K^+	Fe^{2+}	PO_4^{2-}	F^-	$CaCO_3^{2-}$	F Cl_2
T ^{O}C	1												
pH	**.886****	1											
TDS	**-.520***	**-.444***	1										
EC	-.327	-.292	**.865****	1									
LGW	**-.416***	-.370	-.066	.054	1								
HPC	**-.570****	**-.584****	.274	.178	**.464***	1							
SO_4^{2-}	**.528****	**.493***	-.394	-.114	-.108	-.336	1						
K^+	**-.662****	**-.646****	**.717****	**.586****	.289	.336	-.362						
Fe^{2+}	-.151	-.083	**.509***	**.480***	-.132	.296	-.193	.261	1				
PO_4^{2-}	.325	.300	.121	.176	-.181	-.163	.005	-.212	**.494***	1			
F	**.502***	**.427***	**-.539****	-.380	.074	-.341	.185	-.371	-.229	-.074	1		
$CaCO_3^{2-}$	-.364	**-.513***	.364	.189	.137	.204	-.399	**.511***	.095	.009	-.177	1	
F Cl_2	-.369	-.314	.148	.295	**-.751****	**-.502***	.036	.212	.018	-.014	-.156	.008	1

*NB *. Correlation is significant at the 0.05 level ; **. Correlation is significant at the 0.01 level. LGW=Legionella from water; HPC= Heterotrophic plate count; FCl_2= Free residual Chlorine; $CaCO_3^{2-}$=Alkalinity*

Legionella prevalence from this study was 35.3% from ZH and 43.4% from Y12 hospital, which is relatively higher than what were reported 38% from USA by Donohue *et al*.,(2018), 35% from Germany by Arvand *et al*., (2011) , 33.33 from Italy by Laganà *et al*., (2014), 33% from Greece by Fragoua *et al.,* (2012), 31.1% from Iran by Rafiee *et al.,* (2014), 23% from Iran by Asghari *et al.*,(2013), 23.7% from Rome by Alessandro *et al.,* (2015),

However, the findings in this study were relatively lower than the reported values of 93.7% from Bologna Italy by Leoni *et al*., (2005), 85% from Barcelona Spain by Stout and Yu, (2001), 74.8% from Poland by Sikora *et al.,* (2015), 70% from US by Stout *et al*., (2007), 67.1% from Italy by Antonella *et al.,* (2011) and 64% from Iran by Asghari *et al*., (2013), 62.3% from Palestine by Shareef A and Mimi Z, (2008)

Similarly higher level of *Legionella* (41% to 53%) from Germany by Lesnik *et al*., (2016), 40% to 47% from South Africa by Singh and Coogan, (2005), and 12% to 75% by Hall *et al*., (2003) reported *Legionella* contamination in hospitals water lines. Most of the reported higher values were done by advanced modern molecular testing methods than the culture method that was used in this study. Actually, the level of *Legionella* determination in the hospitals greatly depend up on the kind of methodology followed, the nature and complexity of water distribution, the available water quality management options and others.

Water distribution system of hospital building is prone to oppurtunistic pathogens. *Legionella* as one of oppurtunistic perimise plumbing pathogens (OPPPS) grow and multiply in potable water distribution system Falkinham, (2015) and Yocavitch *et al.,* (2017) causing Legionnaires' disease to susceptible people (Vergis *et al.,* 2000). Hospital-acquired Legionnaires' disease has become a global public health issue (Stout and Yu, 2001). And OPPPS in general and *Legionella* in particular is an emergent concern in owners, managers

and occupants of large building water system of hospitals, hotels etc (Falkinham, 2015; Yocavitch *et al.,* 2017 ; Vergis *et al*., 2000). The presence of free living amoeba (FLA) which may favor the growth and evolution of pathogenicity linked to emerging nosocomial bacterial infections by *Legionella,* with 88.7% detection rate was reoprted from hospital water distribution system (Muchesa *et al.,* 2015; Mariam *et al.,* 2013).

The preliminary study of this study confirms the avaiability of *Legionella* from sampled hospitals distribution system in Addis Ababa which may give a clue for potaintility of *Legionella* infection for patients especially susceptible immunocompromised patients (Muchesaa *et al.,* 2015 and Bartley 2017) found in health care institutions. According to CDC vital signs, about 76% of *Legionella* outbreaks in US were health care exposure associated which killed about 25% of the infected patients (CDC, 2017) resulting in more death than travel associated infections.

Potable water is frequently reported as source of exposure (AWT, 2019). The presence of *Legionella* might be associated with presence of stagnant water (not continuously used) in the distribution system, water system which has "dead legs" (dead branches) of the installations and the quality of intake of water and others (Kozioł *et al.,* 2014). Moreover, warm water, low residual chlorine concentration, presence of sludge, scale, rust, algae or slime deposits in water distribution systems supports the growth of *Legionella spp* (WHO, 2011). Another factor may be lack of effective water management operation in hospital buildings (WHO, 2011a). Perhaps little attention might be given to the management of water distribution system in the hospitals and mostly WDS is considered as passive systems of transporting only water from treatment source to consumer's point of use; but it is not (WHO, 2014).

Due to the vulnerability of some patients, hospitals need to have additional treatment either filtration, disinfection or others at the point of entry of external piped supplies (WHO, 2011). Both of the sampled hospitals do not have additional water treatment facilities (Chlorinator) and are using water directly in their complex buildings and water distribution system coming from public water supplier (AAWSA). Water systems that are kept clean and flowing are less likely to support excess growth of *Legionella spp* specific water safety plans incorporating control measures for *Legionella spp* should be developed for large buildings of Hospitals (WHO, 2011).

Properly unmanaged water distribution system may create conducive environments for growth of opportunistic microbes with in the pipe line. The findings in this study in relation to *Legionella* and *HPC* prevalence may indicate low level of awareness about such emerging infections. The presence of *Legionella* species in the hospital water supply suggests that patients in the hospital may be at risk for hospital-acquired *Legionella pneumonia* and calls for the routine implementation of *Legionella* diagnostic tests for patients with hospital-acquired pneumonia (Stout *et al.*, 2007; WHO, 2007 and Vergis *et al*., 2000). It has been documented by WHO (2002) that, the risk of health care-associated infection (HAIs) rates vary from 5% to 10% of all patients admitted to modern healthcare centers in the industrialized world to up to 25% in developing countries which is about 2 to 20 times higher in developing countries than developed countries.

The concentrations of measured physicochemical water quality parameters from two hospitals vary one from another. This might be associated with the nature of the water sources that AAWSA provides to the Addis Ababa city dwellers on one hand and the implementation of chemicals for treatment of potable water on another. The main potable water source is ground

water (63.3%) while 36.72% comes from surface water treatments plants (31.8%) from Legedadi and (4.95%) from Gefersa Figure 11.

In this study the relative impact of water temperature can be observed from Y12H on, negative correlations of water temperature and *Legionella* (r=-0.416) and water temperature with *HPC* (r=-0.570) than water samples from ZH on which Temperature positively correlates with *Legionella* prevalence (r= 0.343).The mean water temperature from Y12H is relatively higher 29.3^{O}C than ZH (27.1^{O}C) Table 18, 19. The observed difference might be attributed to the number and type of water samples analyzed from two hospitals differs widely. Relatively large number of water samples 34 from Y12H analyzed than 23 in ZH. Most of water samples from ZH were cold water samples (70.6%) getting hot water was difficult while the opposite is true for Y12H as the new hospital building has functional hot water boilers (52.2%) Storage water samples also analyzed in Y12H but not in ZH Figure 21.

The relationship of free residual chlorine level with level of *Legionella* and *HPC* from sampled hospital water is presented in Table 18 and 19. Strong negative correlation was observed between Free residual Cl_2 and *Legionella* (r=-0.75); Free residual Cl_2 and HPC (r=-0.50) from samples of Y12 Hospital. Similarly strong negative correlation observed from samples of ZH between available free residual chlorine with *Legionella* and *HPC* levels(r=-0.459 and r= -0.890) respectively. Despite progress in public health and hospital care, infections continue to develop in hospitalized patients and hospital staffs (WHO, 2002). Infections in general might be increased in the future due to the proportion of aging and immune-compromised patients in our population continue to increase. In line with this, providing awareness for staff member, patients and relatives about the emerging issues of pathogens are necessary this finding might be important in this regard.

Moreover, for diagnostic purposes of emerging pathogens, availability of efficient microbiology labs in infection prevention programs is crucial for successful prevention of HAI outbreaks and sporadic cases (Asifa, 2014). Monitoring *Legionella* infection is very significant as new findings prevailed that *Legionella* acquiring resistance to antibiotic is reported (Kannan, 2017). At least the level of disinfection process and effectiveness of potable water distribution system can be assessed by the level of *HPC* count per mL water samples which tells the cleanliness and integrity of the system (WHO, 2011). In line with this, the *HPC* levels from sampled hospital distribution system showed that 5/34 (14.8%) of samples from ZH and 14/23 (60.9%) water samples from Y12H water samples had *HPC* greater than 200CFU/mL Table 15. Indicating the microbial quality, cleanliness (effectiveness of disinfection) and integrity of the water distribution system especially from sampled hospitals Y12H was questionable.

However, most of the physicochemical parameters of the hospitals water measured were within the guideline values of WHO except for the mean Iron level 0.60mg/L (Y12H) and free residual Chlorine with the values 0.16 mg/L from ZH and 0.19 mg/L from Y12H (<0.2mg/L). The Iron level from Y12 hospital water sample exceeds the maximum permissible level 0.3mg/L (CES, 2013). This might be due to from the source (ground, surface water) or the presence of old iron based distribution system. The Ethiopian standard for potable water Iron level exceeding 0.3mg/L may affect the palatability of drinking water and hence efforts has to be made to keep the Iron level below this standard.

The free residual chlorine level from hospital water distribution systems is lower than the WHO's (0.2-0.5mg/L) and Ethiopian standards (0.5mg/L) for potable water. Low level of residual chlorine in water distribution systems may favor the growth and replication of both

opportunistic environmental microbes including *Legionella* and those pathogenic microorganisms, development of biofilm within water system. As WHO (2011), recommends, for normal use, treated water should have between 0.2 to 0.5 mg/L residual chlorine levels at the point where the consumer collects water. The higher level will be close to the disinfection point and the lower level at the far extremities of the supply network. In this study lower level (<0.2 mg/L) of residual chlorine was measured from sampled hospital distribution system Table 14 which may be associated with hospital water safety issues (Brooke and Tara, 2014) and the level of disinfectant concentration in water distribution system should have to be maintained at the regular level to control the possible infections from opportunistic pathogens from water distribution system (Bartley *et al*., 2017 and Muchesaa *et al.,* 2015).

LD infections, might have been basically associated to the manmade environmental niches and changes in human behavior that have led to *Legionella* infection as a new public health risk that can be allied with morbidity and mortality when the infection is not rapidly diagnosed and treated (Mariam *et al.,* 2013 and Julianne *et al.*, 2015). Although the incidence of *Legionella* infection in health care facilities is low, high fatality rate from 38 to 53% reported by (Stout *et al.,* 2011) emphasising the infection as important emerging nosocomial infection in Health care facilities (Ehrhard *et al.,* 2015; WHO,2002).

Further, health care facilities should have practical prospective water safety plans that include preventive measures, which is preferable than remediation of contaminated hospital water distribution system (Brooke and Tara, 2014). One such action to reduce the risk of LD might be implementation of disinfecting treatment (On site disinfection) of water system (Antonella *et al.,* 2011). Continueus monitoring surveillance of microbial condition of water distribution

systems are an important and necessary element of the control of infections caused by *Legionella* and other opportunistic organisms (Sikora *et al.,* 2015; Fragoua *et al.,* 2012 and Antonella *et al.,* 2011).

Hospitals should have to maintain proper monitoring and management of water distribution system since older people with chronic medical conditions, immunocompromized individuals and immunosuppressive transplant recipients are highly vulnerable and have an increased risk of hospital acquired infections (Mercante and Winchell, 2015). Furthermore, additional information is needed for the optimal prevention and control measures, in a healthcare facility; supplemental disinfection of the water distribution system is an operative approach to *Legionella* infection prevention (Lin *et al.,* 2011; Stout *et al.,* 2011and Sikora *et al.,* 2015).

LD resolution requires swift treatment together with rapid diagnosis, accurate antibiotic management and epidemiological awareness (Jeffrey and Ehrhard *et al.,* 2015). Equally, ecological studies on water distribution system on *Legionella spp* are essential to understand better their sources, mechanism and enabling factors of *Legionella* entry into manmade water systems (Mariam *et al.,* 2013). Likewise, there should be shared responsibility in managing the *Legionella* risk in Hospitals in one hand the water supplier has to maintain standardized potable water quality parameters and on the other hand the receivers have so manage their building water system (Bourdon *et al.,* 2019).

4.3 Addis Ababa Water Distribution System /AAWDS

4.3.1. Physicochemical Water Quality of AAWDS Water Samples

A total of 26 water samples from AAWDS distribution system has been collected and analyzed for physicochemical and microbial quality parameters. The mean pH level was 6.97 Mean Iron and potassium level was 0.6mg/L and 1.9mg/L respectively. Lack of sufficient concentration of residual free chlorine (0.18mg/L) was also observed from AAWDS. The mean, minimum, maximum levels of tested physicochemical water quality parameters are presented Table 20.

Table 20 Mean and Range physicochemical water quality parameters from AAWDS [a]

Parameters	Values	AAWDS	WHO	CES (MPL)
WT	Mean ±SD	22.71±1.7		-
	Range	21.3-27.9		
pH	Mean ±SD	6.97±0.8	6.5–8.5	6.5-8.5
	Range	6.2-8.7		
TDS	Mean ±SD	100.0±36.4	1000	1000
	Range	73.8-206.9		
EC	Mean ±SD	146.1±47.7	400-1200	-
	Range	110.1-301.2		
SO_4^{-2}	Mean ±SD	4.5±8.3	500	250
	Range	0.0-34.0		
K^+	Mean ±SD	**1.9±0.4**	50	**1.5**
	Range	0.8-2.5		
Fe^{2+}	Mean ±SD	**0.6±0.2**	**0.3**	**0.3**
	Range	0.3-0.95		
PO_4^{2-}	Mean ±SD	8.6±3.5	-	-
	Range	1.3-15.2		
F^-	Mean ±SD	0.7±0.8	1.5	1.5
	Range	0.03-3.0		
$CaCO_3^{2-}$	Mean ±SD	61.7±37.9	600	200
	Range	25.0-140.0		
Free Cl_2	Mean ±SD	**0.18±0.2**	**0.2 to 0.5**	**0.5**
	Range	0.0-0.8		

[a] *Data reported in mg/L except for pH, EC (mS/cm) and Temperature (^OC); WT=Water Temperature; WHO standards for drinking water quality; ; CES=Compulsory Ethiopian Standard ,Drinking water Specifications; MPL=Maximum Permissible Level*

4.3.2. *Legionella Spp* and *HPC* from AAWDS

Among the tested twenty six water and biofilm samples 8/26 (30.7%) of the samples were positive for *Legionella spp* Table 21. Relatively higher than >1000 CFU/L *Legionella* counts were observed from two (7.7%) samples and 13 (50%) samples contained *HPC* count greater than 200CFU/mL. Though, most of the tested biofilm samples were negative with respect to

Legionella. Table 21 presents *Legionella* and *HPC* levels from Addis Ababa water distribution systems.

Table 21 Prevalence of *Legionella* and *HPC* from AAWDS distribution system, 2018

	Water Sample tested for *Legionella Spp*. CFU/L					Biofilm Sample		Total Positive samples
Sample source	Positive (>10CFU/L)				Negative N (%)	Positive N (%)	Negative N (%)	-
	10-99 CFU/L	100-999 CFU/L	1000-9999 CFU/L	Total N (%)				-
AAWDS (N=26)	3(11.5%)	2(7.7%)	2(7.7%)	**7(26.9)**	13(53.8)	**1(3.8)**	4(15.4)	**8(30.7 %)**
Water Samples tested for *HPC* CFU /mL								
	100-199 CFU/mL	200-299 CFU/mL	300-399 CFU/mL	400-499 CFU/mL	Missing			
AAWDS	9(34.6%)	**10(38.5%)**	**3(11.5%)**	-	4(15.4%)			

Some of mixed and pure colonies of *Legionella* from Addis Ababa water distribution system presented in Figure 25 while the mean of *Legionella* and *HPC* with their Log_{10} expression presented in Table 22

Table 22 Mean± SD *Legionella* and *HPC* counts with Log_{10} expression from AAWDS samples

Microbial parameters	AAWDS (n=26)		
	Mean ± SD	Minimum	Maximum
HPC CFU/mL	183.46±97.65	0.0	370
Log_{10}*HPC* CFU/mL	2.11±0.495	1.0	2.56
LGW CFU/L	258.077±795.99	0.0	3000.0
Log_{10} LGW CFU/L	1.41±.757	1	3.47

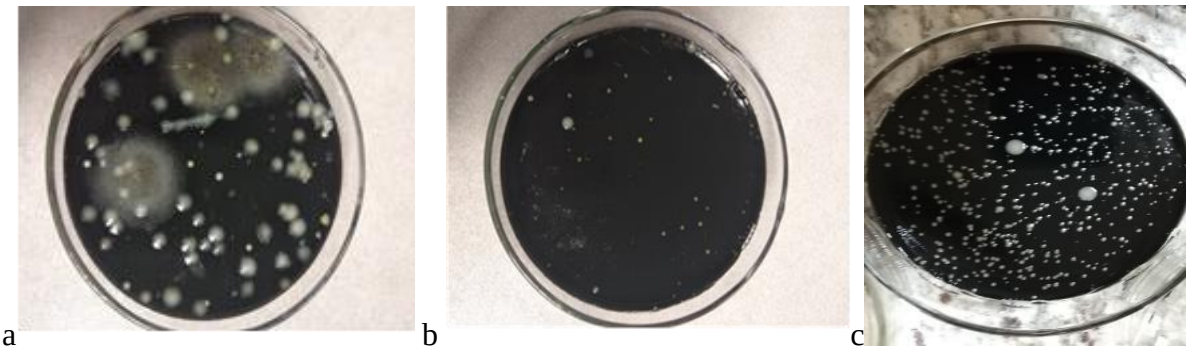


Figure 25 Mixed (a) and pure colonies (b & c) of *Legionella* from AAWDS water distribution system samples on BCYE agar

Of the 26 water and biofilm samples collected from Addis Ababa Water distribution system (AAWDS), 13(50%) exhibited *HPC* greater than 200CFU/mL, while only 8/26 (30.7%) of them were positive for *Legionella spp* Table 21.

Compared to other studies conducted on water distribution system elsewhere, relatively higher level of *Legionella* detection than this study was reported as 87% from Namibia Chinsembu *et al*., (2010), 74.6% from Australia by Hayes *et al.,(*2019) , >69.0% from US by Wang *et al*.,(2012), 50% from USA Donohue *et al.*, (2014), 48% from USA Schwake *et al*., (2016), from Italy by 47.5% Bargellini *et al*., (2011) , 41.7% from Kuwait Qadreyah *et al.*, (2012), 30.7% from Italy by Filippis *et al*.,(2018) and 31.5% Morocco by Mariam *et al.*, (2012).

Comparatively lower level of *Legionella* detection than our finding was also reported as 29% from Italy by Federici *et al.*, (2017), 23.5% from Kuwait Qadreyah *et al.*, (2012) and 16% from US by Donohue *et al.*, (2014). Much lower level of *Legionella* detection reported from water distribution system as 19.5% from Morocco by Jai *et al*.,(2012), 18.62% from Bosnia and Herzegovina by Bešić *et al.,(*2017), 12% from Greece by Dimitriadi and Velonakis

(2014), 11.6% from Gabon by Ehrhard *et al.,* (2015), 11.1% Filippis *et al.,*(2018), 9.4% from Croatia by Rakić *et al.,* (2012) and 8.5% Khaled *et al.,* (2014).

Even if biofilm in water distribution system considered as source of *Legionella* in USA, and Norway reported by Michael *et al.,* (2018) and Filippis *et al*., (2018), in this study most biofilm samples from AAWDS were negative and only 3.8% of them were positve for *Legionella* Table 21. Infact, factors like water stagnation and warmer environmental conditions and others might contirbute for the prevalnce of *Legionella* in biofilms but our finding is not in agreement with the intent that *Legionella Spp* grow and multiply to higher level within biofilms of pipe wall of the sampling point (Ashbolt and Nicholas 2015).

But, the positive water samples from main lines may also indicate *Legionella spp* might come from biofilms that are found far distance from the sampling point. According to Mario *et al.,* (2005) and Flemming *et al*., (2002), biofilms are known reservoir of different kinds of microbes in drinking water supply system and accidental or periodical shading of microbes from biofilms may occur when biofilms reach maturity level. Also being niche for microbes, biofilm aggravates corrosion of iron based water mains, produce bad taste and odor, and decrease residual concentration of disinfectants Michael *et al.,* (2018), the observed low level of mean free residual chlorine 0.18mg/L, higher level of iron (0.6mg/L) and prevalence of *Legionella* (30.7%) might be associated with presence of biofilms in the potable water distribution system.

Addis Ababa has been facing potable water shortage due to rapid population growth, lack of maintenance and new water treatment facilities (UN-Habitat, 2008). Poor environmental conditions, degraded infrastructure (leaky water lines), scheduled water supply and a cross-connected distribution in Addis Ababa may contribute their share for contamination of water

supply (Mekonnen, 2015) including biofilm development, high level of *HPC* per mL and *Legionella* contamination Table 21.

Recently, *Legionella* management in water distribution system has got attention in developed nations Bourdon *et al.,* (2019) but not in developing countries. Education and awareness needed for potable water suppliers, water receivers about risks associated with *Legionella* and others infections. In Ethiopia, there is no any clear picture to how to manage Legionella from water distribution system

The associations of *Legionella* positive samples with free residual chlorine, *HPC* from Hospital and Addis Ababa water distribution system supplied by AAWSA presented in Figure 26.

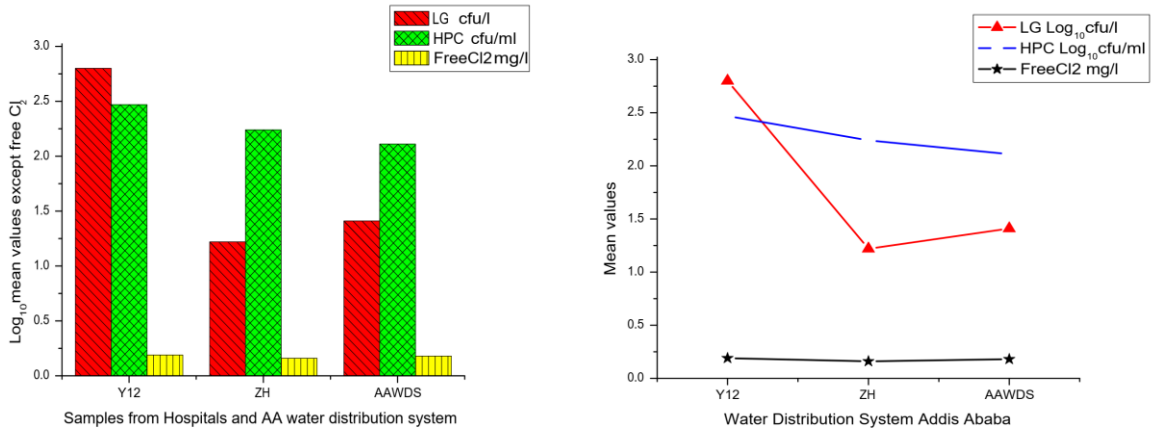


Figure 26 Comparison of *Legionella, HPC* and Free Cl_2 from Sampled AA water

Most LD outbreaks resulted due to process failour, human error and others that can be preventable by effective water management schemes (CDC,2016). Effective potable water management system is not only important to *Legionella* control rather it is an essential

component to SDG6 and for other SDG goals related to health, food and others (UN-Water, 2016).

The tested mean residual chlorine level from AAWDS was (0.18mg/L) which was below 0.5mg/L of ECS standard and between 0.2 to 0.5mg/L of WHO guideline values for potable water. Very low level (0.0mg/L) of residual free chorine compared with AAWDS was reported from 25.7% of the tap water samples of Bahir Dar city, Ethiopia (Milkiyas *et al.*, 2011). Together with poor sanitation, uncontrolled treatment of drinking water quality parameters may contribute to contamination by microbes from the environment and multiplication of opportunistic pathogens in water distribution system.

Thus 30.7% prevalence of *Legionella* and 50% samples with higher level >200CFU/ml of *HPC* detected from AAWDS water samples in this study could be resulted due to low level of free residual chorine in the system. This can be observed from the strong negative correlations at $P<0.01$ between free residual chlorine and HPC ($r=-0.599$) and free residual chlorine and *Legionella*($r=-0.607$). The Level of HPC and Prevalence of *Legionella* from AAWDS was strongly positively ($r=0.859$) correlated at $P<0.001$ therefore *Legionella* survival in AAWDS may be positively influenced by other aquatic germs (Mariam *et al.*, 2013).

The presence of tested microbes indicate the level of deterioration of potable water quality due to chemical and biological reactions in the bulk water and interactions with pipe surfaces USEPA, (2002) or contamination from natural and anthropogenic sources. For efficient utilization of potable water, water quality monitoring, control system, risk assessment and management mechanisms should have to be in place starting from water catchment to point of use (Zinabu and Alemu, 2015).

The pH value of water is more important as operational water quality parameters than its impact on consumers. Drinking water with a pH range of 6.5 to 8.5 is generally considered satisfactory. The measure of the mean pH level in this study was 6.79 and it was almost close to neutral (pH 7) indicating good operational water quality parameter value.

Electrical conductivity (EC) is a measure of the ability of aqueous solution to carry an electric current. This water property depends on the presence and total concentration of dissolved solids (ions), their mobility, valance and on the temperature. EC is crude indicator of water quality and related to TDS in water system. The mean measured EC of AAWDS was 146.1(mS/cm) which is relatively higher than the drinking water quality reported from Bole sub city of Addis Ababa 192.14 μS/cm from Wondogenet by Meride and Ayenew, (2016), 121.26(μS/cm) by Mokenin,(2015) and 65.5(mS/cm) from Nekemete by Gonfa *et al.,* (2019) reported.

The mean TDS level measured from this study was 100mg/L which is below the guideline value levels of both WHO and CES. The finding was relatively lower than the two sources of potable water samples measured from ArbaMinch town as 150 mg/L and 155mg/L by Reda *et al.,* (2015) and Samples from Wondo Genet as 246.8 mg/L by Israel and Awdenegest (2012), but the TDS result of this study much higher than potable water samples measured from Nekemete city as 21.0 mg/L by Gonfa *et al*., (2019). EC and TDS may affect the test or palatability or acceptability of the water. Drinking water that has TDS levels less than 300 mg/L are considered as excellent (WHO, 2003) and hence the analyzed water from AAWDS in this study has such quality. The comparison of the physicochemical quality of potable water this study and other research findings are presented in Table 23.

But TDS level higher than (> 500mg/L) concentrations can contribute to scaling corrosion or encrustation in water pipes and shorten WDS the service life of appliances (WHO, 2003). The impact might be observed further as the corrosion may lead to structural failure, leaks, loss of capacity and deterioration of chemical and microbial water quality (WHO, 2011). Main sources of TDS in drinking water could be from natural sources, sewage, urban runoff and industrial wastewater (WHO, 2011).

The mean Iron level in this study was 0.6mg/L which is beyond the levels recommended by WHO and CES guideline levels. The value is relatively higher than what was reported from previous studies in Addis Ababa (0.07and 0.47mg/L) by Mekonin, (2015) and 0.15mg/L in Wondogent by Israel and Awodenegest, (2012). But lower than the reported drinking water in Nekemete 0.67mg/L by Gonfa *et al.*, (2019). Iron found in natural fresh waters at naturally ranges from 0.5 to 50 mg/L. and in drinking water system source might be the use of iron coagulants or the corrosion of steel and cast iron pipes during water distribution (WHO, 2011).

Table 23 Mean± SD physicochemical parameters of AAWDS compared with Literatures [a]

Locations*	Tem.	pH	TDS	EC	SO_4^{2-}	K^+	Fe^{2+}	PO_4^{2-}	F^-	$CaCO_{3\,2-}$	Free Cl_2	Ref**
AA, ETH	22.7 ±1.7	6.97 ±0.8	100.0 ±36.4	146.1 ±47.7	4.5 ±8.3	1.9 ±0.4	0.6 ±0.2	8.6 ±3.5	0.7 ±0.8	61.7 ±37.9	0.18 ±0.2	This study
NE, ETH	20.9 ± 1.0	6.73 ± 0.1	21.0 ± 2.8	65.5 ± 4.5	17.8 ± 3.4	-	0.67 ± 0.2	0.75 ± 0.14	-	-	0.21 ± 0.2	1
LV, Chad-Cameroon	29.0 ±2.3	6.7 ±0.5	-	243 ±195		-	3.71 ±6.93	0.37 ±0.38	0.17 ±0.22	-	-	2
AM, ETH	-	-	150.76 ± 1.66	-	-	2.88 ± 0.15	-	-	2.048 ± 1.79	666.67 ± 1.5	-	3
AM, ETH	-	-	155.54 ± 1.01	-	-	3.68 ± 0.34	-	-	4.415 ± 0.59	11.11 ± 1.2	-	3
AA, ETH	-	6.97 ±0.39		121.26 ±12.98	-	-	0.07 ±0.08	-	-	-	0.12 ±0.1	4
AA, ETH	-	7.08 ±0.28		136.70 ±14.85	-	-	0.47 ±0.08	-	-	-	0.47 ±0.08	4
WG, ETH	24.3 ±2.9	6.8 ±0.5	246.8 ±248.6	-	-	-	0.15 ±0.26	-	0.50 ±0.39	85.7 ±47.5		5
WG, ETH	28.49		118.19	192.14	0.33	23.14						6
WHO	-	6.5–8.5	1000	400-1200	500	50	0.3	-	1.5	600	0.2-0.5	
CES	-	6.5-8.5	1000	-	250	1.5	0.3	-	1.5	200	0.5	

[a] *Data reported in mg/L except for pH, EC (µS/cm) and Temperature (^OC); * AA, Addis Ababa; AM, Arbaminch ; ECS, Ethiopian Compulsory Standard; ETH, Ethiopia ; LV, Logone Valley ; NE, Nekemtea; WG, Wondogenet; WHO, World Health Organization . ** 1: Gonfa, (2019); 2: Sabrina,(2013); 3: Reda,(2015); 4 : Mekonnen,(2015); 5: Israel and Awdenegest ,(2012);6: Meride and Ayenew, 2016*

Groundwater may contain ferrous iron at concentrations up to several mg/L WHO, (2011), soil leaching and water-rock interaction Sabrina *et al*., (2013). Higher level of iron in water system can result objectionable reddish-brown colour to the water and affect the aestethic quality of the water. The presnece of Iron in water distribution system promotes the growth of "iron bacteria" and involve in the deposit a slimy coating on the piping furhter noticable iron test observed and iron stains laundry and plumbing fixturesif the when the concentration is > 0.3mg/L. Therefore, Iron is not of health concern but at levels causing acceptability problems in drinking water (WHO,2011)

The mean Potassium level reported from AAWDS was about 1.9mg/L which is higher than the recommended in Ethiopian drinking water standard (<1.5mg/L). Higher level than this study was reported from two drinking water source of ArbaMinch as 2.88 mg/L and 3.68mg/L (Reda, 2015). Source of the potassium ion in drinking water might be from nature or from water treatment procedures on which potassium permanganate used as an oxidant in water treatment (WHO, 2003). Potassium may cause some health effects in susceptible individuals or high-risk groups (i.e. individuals with kidney dysfunction or other diseases, such as heart disease, coronary artery disease, hypertension, diabetes, adrenal insufficiency (WHO, 2011).

Sulfate level measured in this study was 4.5 mg/L which is lower than Sulfate level measured from Nekemete 17.8 mg/L (Gonfa *et al.*, 2019). Both of them were below the guideline value for drinking water. According to WHO (2011) gastrointestinal / health effects observed from ingestion of drinking-water containing high sulfate levels (>500mg/L). Besides this sulfate also changes the test of the water and may contribute to the corrosion of pipe line in distribution systems. The main source for sulfate could be ground water (naturally) as main source of Addis Ababa water (63.2%) atmospheric depositions, geologic/minerals and biological process, industrial discharges since surface water reservoirs are used as additional water sources (WHO, 2004; ESEPA, 2003).

4.3.3 Associations of Microbiological Parameters and Abiotic Factors

The microbial and physicochemical water quality parameters tested from AAWDS had different level of correlation as shown in Table 24. Positive correlation at $P<0.01$ were observed between *Legionella* and *HPC*($r=0.859$) , between EC and TDS ($r=0.997$), PO_4^{2-} and EC ($r=0.665$), F and SO_4^{2-} ($r=0.535$), F and PO_4^{2-} ($r=0.58$), F and pH ($r= 0.521$) Similarly,

positive correlation was observed between $CaCO_3^{2-}$andTDS (r=0.519), $CaCO_3^{2-}$ and EC (r=0.515), $CaCO_3^{2-}$ and PO_4^{2-} (r=0.545), $CaCO_3^{2-}$and F (r=0.661). On contrary negative correlation at p<0.01 were observed between free residual chlorine and *Legionella* (r= -0.607), Free residual chlorine and HPC (r= -0.599) Table 24.

Table 24 Spearman's rho correlation of physicochemical and microbial quality of AAWDS, 2018

AAWDS	HPC	LGW	pH	TDS	Temp	EC	SO^{2-}_4	K^+	Fe^{2+}	PO^{2-}_4	F^-	$CaCO^{2-}_3$	FRC
HPC	1												
LGW	**.859****	1											
pH	.015	-.031	1										
TDS	-.189	-.386	0.323	1									
Temp	.199	.102	0.152	0.097	1								
EC	-.185	-.379	0.334	**.997****	0.09	1							
SO^{2-}_4	.164	.233	0.386	0.26	**.474***	0.257	1						
K^+	-.021	.051	0.108	0.135	-0.127	0.14	0.15	1					
Fe^{2+}	.158	.052	-0.155	0.112	-0.322	0.089	0.138	-0.117	1				
PO^{2-}_4	.137	.051	0.377	**.671****	0.091	**.668****	**.440***	-0.001	0.21	1			
F^-	.040	.079	**.521****	0.288	0.281	0.287	**.535****	**.443***	-0.103	**.528****	1		
$CaCO^{2-}_3$	-.279	-.331	**.485***	**.519****	0.068	**.515****	0.239	**.415***	-0.129	**.545****	**.661****	1	
FRC	**-599****	**-.607****	-.018	.253	-.180	.404	-.206	-.290	.244	.033	-.089	.247	1

[a] *Data reported in mg/L except for pH, EC (mS/cm) and Temperature (^OC); LGB, Legionella from biofilm; LGW, Legionella from Water; FRC, Free residual Chlorine ;**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).*

Sub-Saharan Africa is urbanizing faster than any other continent (WHO&UNICEF, 2014). With main challenges of water quality and sanitation aspect, the ever growing city population is sharing poorly managed and inefficient water bodies. Similar trend of urbanization is observed in Ethiopia where an average growth rate of 5.84% has been observed for last fifty years (1961-2013), which has resulted with about 755.6 people adding to the urban residents

daily (MoUDC,2014). The urban population is now expected to double in the next two decades to reach 30.1% by the year 2020 (Abebe *et al.,* 2015).

In addition lack of adequate water in urban areas, the quality potable water also being challenged by emerging opportunistic pathogens. Due to lack of awareness about potential risks and lack of skills on training of staff and managers on drinking water systems may results in outbreaks of waterborne diseases in the community (Hrudey and Hurdy, 2014). The realization of the existing prevailing conditions coupled with a preventative rather than just responsive management approach is important (Ashbolt and Nicholas 2015).

Properly managed water distribution system (WDS) can protect drinking water system from contamination. Little attention is given to management of water distribution system and mostly WDS are measured as passive systems of transporting only water from treatment source to consumer's point of use but it is not (WHO, 2014). Countries of growing economies like Ethiopia should consider such emerging water distribution problems and develop strategies of monitoring and maintains of water distribution system. Control strategies for WDS are required including maintaining proper amount of disinfectant level in the system Mario *et al.,* (2005), monitoring opportunistic pathogens in water distribution mains and consumers plumbing system Rakić *et al.,* (2012) and for the development of active prevention mechanisms of possible infections (Mariam *et al.,* 2012).

Mostly, drinking-water safety is ensured by good management practices (Water safety plan), including sound design, routine maintenance protocols, regular cleaning, temperature and flow management like avoidance of stagnation (WHO, 2011).

4.4 Natural Water Bodies (NWB)

4.4.1 Physiochemical Parameters of Natural Water Bodies

The mean Temperature of natural water bodies ranged from 18.6^{o}C at LTA to 25.2 ^{o}C AR. Mean Water temperature were relatively greater than 20^{o}C in all of sampled natural bodies except LTA which was about 18.6±1.1^{o}C. The mean pH was between 6.6 (LTA) to 8.5 (LHO). While mean TDS was between 126.1mg/L (LDA) to 1369.8 mg/L (LHO). Electrical conductivity was measured were greater than 800(µS/cm) from Rift Valley Lakes of LHA, LBA and LHO. The mean physical water qualities if tested natural water bodies presented in Table 25.

Among the natural and artificial Lakes, the minimum sulfate level were 2.20 mg/L from LHO whereas the maximum 37.8mg/L at LDA. The highest concentration of Potassium was measured from LHA 44.8mg/L whereas the minimum was from LTA 2.38mg/L Table 26 below shows the mean tested chemical parameter for natural water bodies.

Table 25 Physical water quality parameters from Natural water bodies, Ethiopia 2018

Parameters	Sampling points					
	LBA	LHO	LHA	LTA	LDA	AR
WT						
Mean(SD)	24.2(2.1)	23.8(2.5)	22.2(2.4)	18.6(1.1)	20.9(2.6)	25.2(0.4)
Range	22.4-26.9	21.7-27.0	20.1-25.5	16.7-19.6	18.7-23.9	24.6-25.6
pH						
Mean	8.4(0.3)	8.5(0.4)	8.2(0.2)	6.6(0.3)	8.1(0.5)	7.5(0.8)
Range	8.06-8.9	8.11-9.30	8.04-8.4	6.3-7.05	7.5-8.5	6.2-7.98
TDS						
Mean(SD)	593.1(63.5)	1369.8(54.4)	593.3(31.3)	255.9(33)	126.1(45.2)	285.4(10.7)
Range	530.0-711.2	664.7-1733	546.8-612.1	103.4-851.4	75.6-167.0	273.1-299.7
EC						
Mean(SD)	845.5(92.3)	1813.7(714)	816.4(37.1)	356.3(447)	182.8(62.8)	409.6(13.3)
Range	761.4-1005.7	888.2-2288.0	761.9-842.5	151.9-1150.0	112.0-238.3	392.6-423.3

***NB**: - WT= Water Temperature; LBA= Lake Babogaya; LHO=Lake Hora; LHA= Lake Hawassa; AR= Awash River; LTA= Lake Tana; LDA= Legedadi Dam reservoir; [a] Data reported in mg/L except for pH, EC (µS/cm) and Temperature (^{O}C)*

Maximum Iron (Fe^{2+}) level was measured from LHO (8.4mg/L) and the minimum was from LTA (0.38mg/L). The maximum fluoride level was measured from LHA (7.1mg/L) while the minimum level measured from LTA (0.24mg/L). The minimum Alkalinity level was measured from LTA as (74mg/L) while the maximum was from LHO (556.7mg/L) Table 26.

Table 26 Chemical water quality parameters of Natural water bodies, Ethiopia [a]2018

Parameters	Sampling points					
	LBA	LHO	LHA	LTA	LDA	AR
SO_4^{2-}						
Mean(SD)	3.2(0.8)	2.2(1.8)	11.8(5.6)	3.20(1.30)	37.8(21.04)	48.0(17.9)
Range	2.0-4	0.0-4	8.0-20.0	1.0-4.0	15.0-66.0	40.0-80.0
K^+						
Mean	18.8(7.5)	22.2(11.5)	44.8(40.3)	2.38(0.16)	13.6(20.4)	9.5(2.1)
Range	12.0-30.1	3.1-34.1	21.1-105.0	2.20-2.60	2.1-50.1	8.0-13.1
Fe^{2+}						
Mean(SD)	3.7(5.1)	8.4(4.8)	1.6(1.8)	0.38(0.31)	3.62(1.6)	7.9(3.3)
Range	0.3-11.1	2.15-12.5	0.45-4.25	0.05-0.70	1.5-5.3	4.5-11.1
PO_4^{2-}						
Mean(SD)	20.7(5.9)	24.1(3.9)	28.2(22.6)	4.95(1.49)	48.3(32.5)	27.6(19.7)
Range	12.1-26.1	18.3-28.0	15.1-62.1	3.40-6.54	4.5-82.4	9.1-57.0
F^-						
Mean(SD)	2.7(4.1)	2.2(2.2)	7.1(0.6)	0.24(0.09)	1.88(2.9)	2.4(0.7)
Range	1.04-10.8	1.26-6.8	6.5-7.8	0.14-0.34	0.01-6.7	1.3-3.1
$CaCO_3^{2-}$						
Mean(SD)	465.0(91.2)	556.7(126.1)	377.5(25.1)	74.1(7.42)	257.8(63.5)	300.1(183.7)
Range	420.1-650.1	400.1-700.1	350.1-410.1	65.1-85.1	150.1-299.1	150.1-600.1

NB:- LBA=Lake Babogaya; LHO= Lake Hora; LHA= Lake Hawassa; AR= Awash River; LTA= Lake Tana; LDA=Legedadi dam reservoir . [a] *Data reported in mg/L*

Mean higher level of Sulfate, Phosphate, Potassium, Iron and Flouride was measured from AR, LDA, LHA, LHO and LHA respectively. The mean differences of physicochemical parameters tested from selected natural water bodies presented on Figure 27.

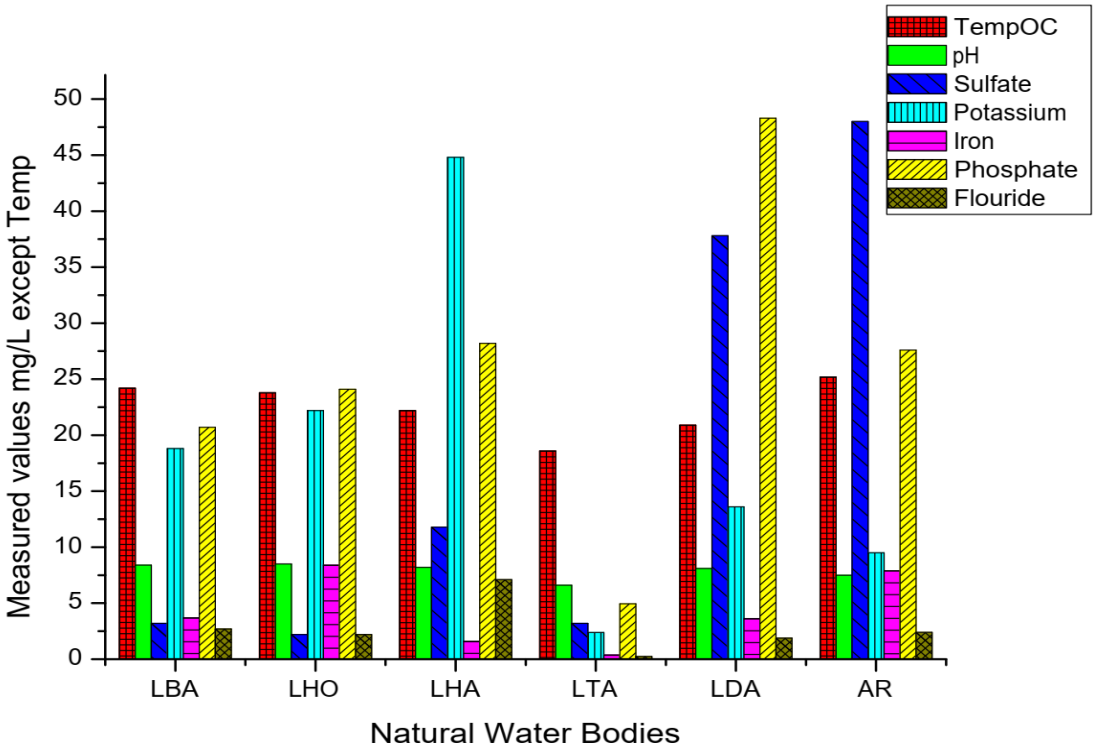


Figure 27 Mean values of physicochemical parameters tested from Natural water bodies

Positive relations observed from TDS and EC from sampled natural hot springs. Except the level of Alkalinity Legedadi Dam reservoir (LDA) has relatively the lower level of TDS, EC than others while Lake Hora (LHO) has got relatively higher level of TDS, EC and alkalinity than others sampled natural water bodies Figure 28

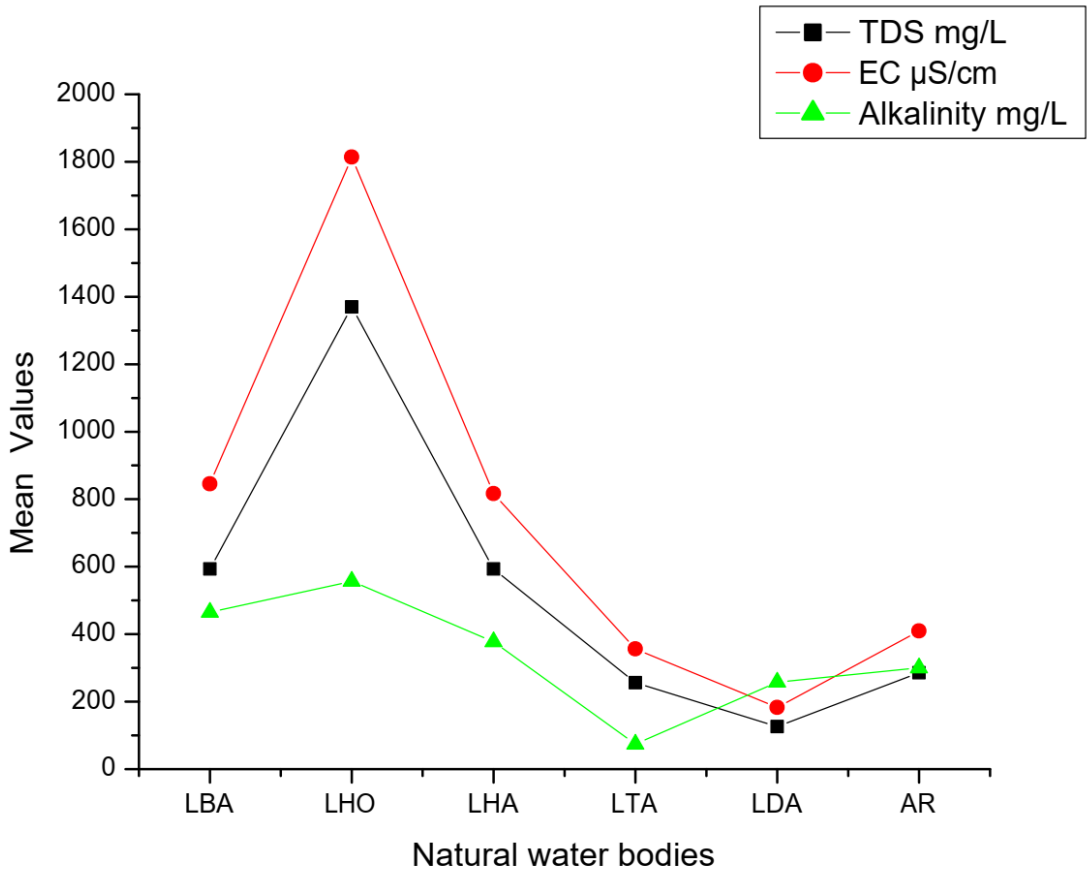


Figure 28 Mean values of TDS, EC and Alkalinity from natural water bodies

4.4.2 The *HPC* and *Legionella spp* from Natural Water Bodies

4.4.2.1 *Legionella spp* from Natural Water Bodies

Legionella was isolated from 61.3% (19/31) natural water samples. Specifically, *Legionella* was isolated from Almost half of sampled natural Lakes (LBA, LHO, LHA, LTA) 10/21 (47.6%) *Legionella* detected. In line with this, 80% positive samples observed from LDA, an artificial Lake or dam reservoir, for drinking water supply to Addis Ababa and all of the samples from Awash River were positive for *Legionella.* The detection level of *Legionella*

from sampled natural water bodies is presented in Table 27. Some of the *Legionella* colonies found from sampled natural water bodies are presented in Figure 29.

Figure 29 Some of Isolated *Legionella Spp* from Natural water bodies, 2018

Higher level of *Legionella* detected from sampled Ethiopian natural water bodies. Mean Log_{10} expressions of *Legionella* was AR (3.29) >LDA (2.34) > LTA (1.82) > LHA and LBA (each 1.71) > LHO (1.66) Table 27.

Table 27 Level of detection and Log_{10} expression of *Legionella* fromNWB, Ethiopia 2018

Sample source	**Water Sample tested for *Legionella***					**Log_{10} expression of *Legionella***		
	Positive (>10CFU/L)				Negative			
	10-99 CFU/L	100-999 CFU/L	1000-9999 CFU/L	Total (%)	No (%)	Mean ±(SD)	Min	Max
LBA (N=6)	-	3 (50%)	-	**3 (50)**	3(50)	1.71(0.82)	1.00	2.90
LHO (N=6)	1 (16.7%)	2 (33.3%)	-	**3 (50)**	3(50)	1.66(0.82)	1.00	2.95
LHA (N=4)	-	2 (50%)	-	**2 (50)**	2(50)	1.71(0.83)	1.00	2.54
LTA (N=5)	-	1 (20%)	1(20%)	**2 (40)**	3(60)	1.82(1.16)	1.00	3.49
LDA (N=5)	-	2 (40%)	2(40%)	**4 (80)**	1(20)	2.34(1.01)	1.00	3.46
AR (N=5)	-	1 (20%)	4(80%)	**5 (100)**	-	3.29(0.31)	2.97	3.72
Total (n=31)	1(3.2%)	11 (35.5%)	7(22.6%)	**19 (61.3)**	**12 (38.7)**			

NB:- LBA, Lake Babogaya; LHO, Lake Hora; LHA, Lake Hawassa; AR, Awash River; LTA, Lake Tana; LDA, Legedadi dam reservoir

4.4.2.2 *HPC* Enumeration from Natural Water Bodies

Consortia microbial population was observed from natural water bodies and about 87.09% of tested samples had more than $2X10^3$CFU/mL. The mean Log_{10} *HPC* level observed at LDA was 3.32 whereas the mean Log_{10} from AR was 3.79. The detection level of *HPC* from sampled natural water bodies is presented in Table 28.

Table 28 Level *HPC* and Log_{10} expression from NWB, Ethiopia 2018

Sample Source	***HPC* CFU/mL**					***HPC* Log_{10}**		
	500-1000	>1000-1500	1500-2000	>2000-2500	>2500	Mean(±SD)	Min	Max
LBA(N=6)	1(16.7%)		1(16.7%)	1(16.7%)	3(50%)	3.44 (0.283)	2.99	3.73
LHO(N=6)	-		1(16.7%)	1(16.7%)	4(66.7%)	3.48 (0.149)	3.26	3.61
LHA(N=4)	-	-	-	-	4(100%)	3.48 (0.048)	3.43	3.54
AR(N=5)	-	-	-	-	5(100%)	3.79 (0.056)	3.72	3.85
LDA(N=5)	-	1(20%)	-	2(40%)	2(40%)	3.32(0.167)	3.06	3.51
LTA(N=5)	-	-	-	-	5(100%)	3.57(0.052)	3.52	3.65
Total 32	1(3.22%)	1(3.22%)	2(6.45%)	4(12.9%)	23(74.19%)			

NB:- LBA, Lake Babogaya; LHO, Lake Hora; LHA, Lake Hawassa; AR, Awash River; LTA, Lake Tana; LDA, Legedadi dam reservoir

Relatively higher level of *HPC* per ml counted from samples of natural water bodies. Some of counted *HPC* colonies are presented in Figure 30.

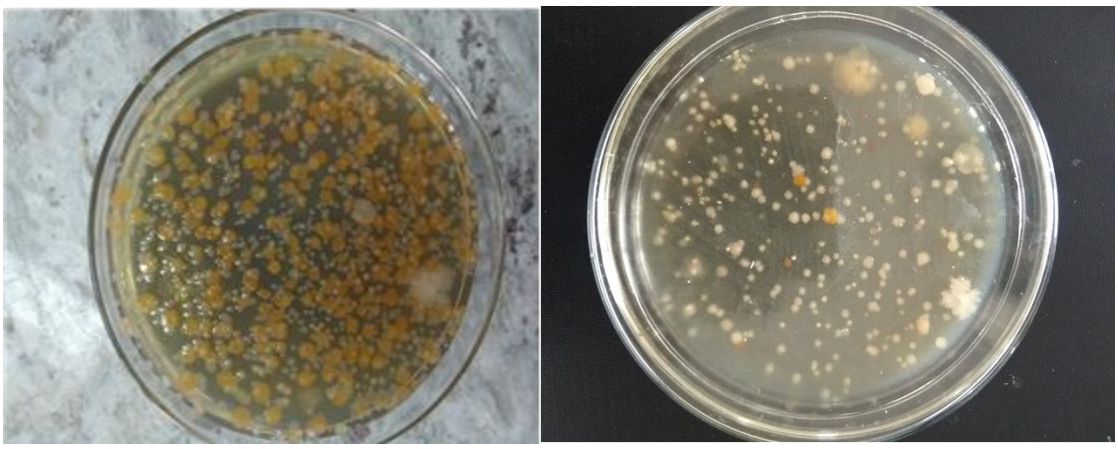


Figure 30 *HPC* from NWB CFU/mL, spread plate method, 35°C/48 h plate count agar 2018

The microbial parameters relationships of *HPC* count with *Legionella* in Log_{10} scale presented on Figure 31.

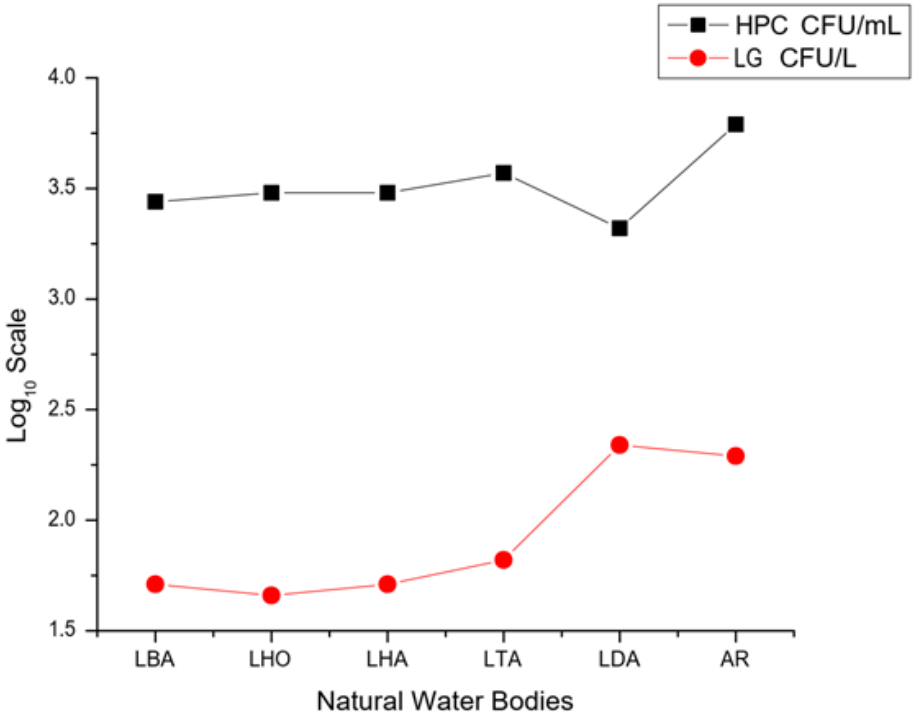


Figure 31 Mean Log_{10} scale relations of *HPC* and *Legionella* from natural water bodies

Among the tested 31 water samples from the natural water bodies 19 (61.3%) of them were positive for *Legionella Spp* specifically, *Legionella* were less in fresh alkaline Lakes 47.6% of samples from natural Lakes (LBA, LHO, LHA, and LTA); than 80% of Legedadi reservoir and all of Awash River (AR) samples were positive for *Legionella* Table 27. As it has been described by Borges *et al.,* (2012), freshwater environments are major reservoirs of *Legionella spp* and found in watery soils, natural or manmade aquatic environments.

In this study all of the water samples from Awash river (AR) were 100% positive for *Legionella spp.* This value is much more higher than *Legionella* diversity and abundance, reported from samples of rivers 52.3% by Hsu *et al.*,(2015), 20.8% by Parthisot *et al.*, (2010), 8% Terry *et al.*, (2011), 1% by Kannan *et al.*,(2017). Awash River (AR) is one of the longest inland river that recieves water from different water shades, of agriculture, industrial, urban and rural areas. *Legionella* prevalence reported from watersheds by Peabody *et al.,* (2017). All samples from two Taiwan Rivers were positive for *Legionella* (Shen, *et al.*, 2015). It is belived that the physicochemical quality and microbial qaulity may favour for the growth of *Legionella* and other types of organisms.

In this study higher level (50%) *Legionella* detected from three rift valley lakes (LHO, LBA, and LHA) but relatively lower level from Lake Tana (40%). All of Lake Samples were higher than *Legionella* prevalence reports as 20% from Lakes ,10% from Ponds by (Kannan *et al.,*2017). More over, in this study *Legionella* were isolated 80% from Legedadi reservoir (LDA) which is higher than 75% from Namibia dam reported (Chinsembu *et al.,* 2010).

The findings of this research are somewhat higher to certain extent than most available research reports. This might be due to limited number of water samples analyzed, sampling season, the existed environmental condition, physicochemical conditions, methodological

approaches followed and others vary greatly and results different level of detections. Most of our sampling was done during the dry season and *Legionella* isolation and prevalnce relatively higher in dry and hot (summer) seasons compared with the winter(rainy season) (Kannan *et al*., 2017). Temperature is a factor that affects the motility, and virulence nature of *Legionella*, the mean temperature from the study Lakes except LTA were above $20^{O}C$ indicates another physical environment for *Legionella* growth from Ethiopian water bodies (WHO, 2007).

Majority of studies have focused on the source of *Legionella* contamination in the manmade systems than the natural water environment where significant concentration may present than previously thought. Parthuisot *et al*., (2010), emphasized the importance of *Legionella* study from natural environment to understand the origin and impact of *Legionella spp* in environmental and anthropogenic effects. Further, to control possible *Legionella* infection surveying and monitoring *Legionella* from aquatic environment has vital importance (Terry *et al*., 2011).

Hence, knowledge of the opportunistic pathogens, level of contamination in various environments is an essential to raise awareness, preparedness, prevention and management of future outbreaks of LD (Chinsembu *et al.,* 2010). However, regarding published articles, there are limited published reports from African aquatic environment excluding reports from South Africa, Nigeria, and Namibia (Terry *et al*., 2011; Chinsembu *et al.,* 2010) and hence this research finding is a pioner in reporting *Legionella spp* prevalence from Ethiopian water bodies and important in filling the existing gap and providing additional knowlge to the scienctific community.

Water quality refers to the physical chemical and biological quality characteristics of water that influence its suitability for specific use. Good quality water is very important for general use, drinking, cooling, cleaning, irrigated agricultural crops, washing and processing equipment's. Rim, (2013) reported lack of resources accessibility about surface water bodies in Africa. Similarly, because of the scarce documented resources available about the physicochemical biological qualities of Ethiopian fresh water bodies, comparing the results of our finding to the others is difficult.

Besides the lack of strong correlation, the trend observed between the level Iron with positive *Legionella* were similar. Iron level was relatively higher at AR, LH and LBA while least at LTA. The Number of *Legionella* postive samples with the level of Iron (Fe^{2+})mg/L presented in Figure 32.

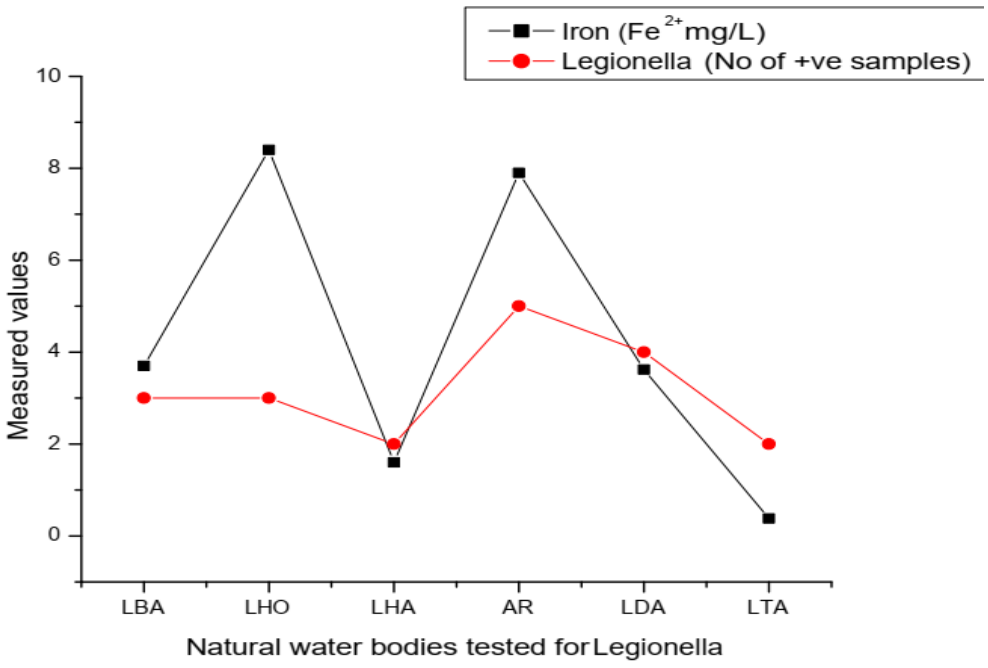


Figure 32 Mean (Fe^{2+}) level with number of *Legionella* Positive samples from Natural water bodies

Legedadi reservoir (LDA) The mean pH of LDA relatively alkaline than Opa reservoir (7.4) in Nigeria (Akindele and Adeniyi 2013), Bira dam (7.02) in Ethiopia (Tesema, 2014) but water samples from Bira dam at Bati Woreda, Amhara region has higher electrical conductivity (399.0µS/cm) than LDA (Tessema, 2014). The level of total alkalinity, Phosphate, Iron, Potassium, Sulfate, Electrical conductivity and TDS from LDA Table 29, water samples were higher than Opa reservoir in Nigeria by (Akindele and Adeniyi 2013). Total dissolved solids, Electrical conductivity and total alkalinity were comparatively higher in rift valley lakes (LBA, LHO and LHA) Figure 28 than LTA and LDA. Mainly the environmental conditions, geology and anthropogenic activities may influence the nature of water quality on sampled area. This might be associated to the water quality difference due to human factors in combination with the geology, warm climate, and physiographic factors such as rugged terrain, through effects on rates of mineralization, soil erosion and transport of particulates and solutes (Zinabu *et al*., 2002).

Table 29 Mean ±SD physicochemical parameters of LDA compared with literatures

Locations	Tem.	pH	TDS	EC	SO_4^{2-}	K^+	Fe^{2+}	PO_4^{2-}	F^-	$CaCO_{3-}^{2-}$	Ref.**
LDA	20.9± 2.6	8.1± 0.5	126.1± 45.2	182.8± 62.8	37.8±2 1.04	13.6±2 0.4	3.62± 1.6	48.30± 32.5	1.88±2. 9	257.80± 63.5	1
OR	26.3 ±1.7	7.38 ±0.36	111±5 3	161.9 ±76.5	1.5 ±1.4	4.36±2 .52	1.7±6 .04	0.49± 0.51	-	72 ±47.9	2
L&D	20.98 ±3.3 1	7.54 ±0.6	74.26± 59.41	139.09± 124.5	-	18.73 ±3.73	14.99 ±3.6 8	0.63±0. 26	0.18±0. 07	-	3

[a] *Data reported in mg/L except for pH, EC (mS/cm) and Temperature (^OC);LDA=Legedadi dam reservoir; OR, Opa reservoir(Nigeria); L&D Legedadi and Dire* catchments

NB: **:- 1: This study; 2: Akindele and Adeniyi, (2013), 3: Yilikal Anteneh *et al.,* (2018)

The presence of *Cryptosporidium oocysts* and *Gardia cycsts* has been reported from Legedadi dam reservoir (LDA) indicating the prevalence of parasitological importance protozoa species in the reservoir (Nigus *et al*., 2008). As part of the life cycle *Legionella* has a capacity to parasitize free living amoeba protozoa families. There might indicate the possibility of getting both protozoa and *Legionella* in water reservoirs for drinking water treatment facilities.

Awash River Awash River is one of highly utilized river especially for agriculture. Since downstream of Awash River water is being used for various purposes such as drinking water supply (Adama City) and irrigation; public health risks are high, in the urban and rural area. The measured pH value from this study was 7.5 which is relatively close to the pH values of Akaki River and Awash River near Wonji (7.8) as reported by Sisay *et al*., (2017) and Ruffeis *et al.*,(2010) respectively. Similar range of pH was registered from Aboabo River (Ebenezer *et*

al., 2014). But Temesgen and Seyoum (2018), reported pH values (6.08 to 8.47) with lower mean pH values (7.23) than this study from Awash River.

The TDS level from Awash River (285.4mg/L) is much lower than River Aboabo (1005-3568mg/L Ebenezer *et al*., (2014) and Akaki River 463.86mg/L Sisay *et al*.,(2017). The mean measured EC level of Awash River (409.6 µS/cm) is higher with the same place with different sampling point (Wonji) as 293-296 µS/cm Ruffeis *et al*.,(2010) reported but lower than Akakai river, 663.23 µS/cm Sisay *et al*., (2017) and 536.76 µS/cm with considerable changes 286.17 to 721, 43 µS/cm Temesgen and Seyoum (2018), along different sampling points of Awash River. The comparison of some of physicochemical qualities of Awash River and others are compared in Table 30.

The Iron level in the Awash River is about 8mg/L which is higher than Akaki river 5.89 mg/L Sisay *et al*., (2017), dry season samples of Awash river 2.17mg/L(Temesgen and Seyoum, 2018), Warri River(1.9mg/L) in Nigeria (Macdonald, 2011). While the Potassium level at Awash River was about 9.5 mg/L which is higher than 0.23mg/L reported from Awash River near Wonji by Ruffies *et al*., (2010).

The Phosphate level from Awash River was about 27.6mg/L which higher than Akakai river (0.28mg/L) as reported by Sisay *et al.,* (2017). Sulfate level from Awash river determined as 48.0mg/L which is much lower than range values of Aboabo River (312-411mg/L) reported by (Ebenezer *et al*., 2014). These are chemical substance added to the river from nature as well as anthropogenic activities from industries, agricultural and others.

The Fluoride levels of Awash River measured from two points were 1.3 and 0.9 mg/L (Ashley and Burley 1994) this was relatively lower than our finding 2.4mg/L. Sulfate source

associated with atmospheric, geologic and biological process origins. Moreover Sulfate might be discharged to surface water through industrial waste (WHO, 2004; ESEPA, 2003). Awash River (AR) is one of the longest inland river that recieves water from different water shades, of agriculture, industrial, urban and rural areas

Table 30 Mean± SD physicochemical parameters of Awash River compared with Literatures [a]

Rivers	Tem.	pH	TDS	EC	SO_4^{2-}	K^+	Fe^{2+}	PO_4^{2-}	F^-	$CaCO_{3-}^{2-}$	Ref**
AR	25.2±0.4	7.5±0.8	285.4± 10.7	409.6± 13.3	48.0±17.9	9.5±2.1	7.9±3.3	27.6±19.7	2.4± 0.7	300.0± 183.7	This study
HR	22.4-23.0	7.91 - 8.18	113.8-382.5	175.1 - 588.4	22.4-40.1	-	-	0.28 - 1.58	-	48- 91	1
ALR	22.5-23.0	7.26 - 8.27	323.1-512.5	497.0 - 788.4	28.1-55.9	-	-	0.43-1.75	-	78- 140	1
NRR	21.21±0.16	6.76± 0.07	142.59± 22.68	109.6± 2.85	-	-	-	55.0±8.52	-	-	2
ER	-	-	700.2 - 1328.2	904.11 - 2156.11	271.82 - 384.07	-	-	0.04 - 0.19	-	131.85 - 267.26	3
TAR	20.9±2.	7.18± 0.07	1027±32	1533±48	519±6	-	-	0.07±0.00 to	-	-	4
TAR	23.6±3.1	8.13± 0.09	1255±33	1874±50	921±22			0.58±0.38			4
GR		8.44	149.37 ± 20.64	316.47 ± 72.802		8.51 ± 0.33		3.50 ± 0.32		154 ± 15.556	5
AR	22.2	7.23		536.76			2.17				6

*NB: [a]Data reported in mg/L except for pH, EC (µS/cm) and Temperature (^OC); L[b] AR, Awash river(Ethiopia); HR, Huluka river (Ethiopia), ALR, Alaltu river (Ethiopia);NRR, Nyanchwa-Riana River(Kenya); Elala River(Ethiopia); TAR, Tseada Agam River(Ethiopia);GR, Guder River(Ethiopia); Ref**,1= Prabu et al.,(2010); 2= Ogendi, (2015); 3= Ftsum,(2015); 4=Kidu,(2015); 5= Olbasa, (2017), 6=Temesgen and Seyoum,(2018)*

Rift valley Lakes As Rim (2013) explained, studies on the physicochemical and biological composition of stagnant surface water bodies such as ponds and Lakes are still scarce and limited. The same scenario exists in Ethiopian fresh water bodies. Among the creator Lakes found at Bishoftu (Debre Zeit), from Lake Hora Kilole, Brook, (2003) reported conductivity

as 339 µS cm^{-1} which is much lower than the conductivity measured from this study from lake Hora Arsedi (1813.7 µS cm^{-1}) Lake Babogaya (845.5 µS cm^{-1}). The mean temperature range (19.3-24) and pH (7.2-9.2) reported from Hora Kilole (Brook, 2003). Moreover, Thewodros and Seyoum (2014) reported the pH and temperature from Lake Hora Arsedi (24.1^{O}C and 8.83) and Babogaya (24.2^{O}C, and 8.9) respectively Table 31.

From this study the mean Fluoride level highest at Lake Hawassa and least from Lake Hora (LHA>LBA>LTA>LHO) decreasing order. Others available literature reported the fluoride level from Hawassa Lake as higher 12.8mg/L (Admasu *et al*., 2015) and 9.0 mg/L (Tenalem, 2009). This might be associated with the geographical location of the Lake (main rift valley) on which higher level of fluoride greater than 10 mg/L expected in waters from the Rift and recognized water-related health concern in Ethiopia (BGS, 2001). Fluoride may also found in excess of 200 mg/l in some of Ethiopia's alkaline, saline Lakes Chitu, Shalla and Abayata (Gizaw, 1996).

The other physicochemical characteristics of sampled lakes indicates that Iron level in decreasing order were higher at LHO and the least from Lake Tana (LHO>LBA>LHA>LTA). Except Lake Tana (pH 6.6), most sampled Lakes had alkaline pH, LHO (8.5), LBA (8.4), LHA (8.2) Comparison of tested physicochemical parameters with available literatures are presented in Table 31.

Table 31 Mean ±SD Physicochemical parameters of sampled Lakes compared with Literatures [a]

Locations *	Tem.	pH	TDS	EC	SO_4^{2-}	K^+	Fe^{2+}	PO_4^{2-}	F^-	$CaCO_3^{2-}$	Ref**
LHA	22.2± 2.4	8.2± 0.2	593.3± 31.3	816.4± 37.1	11.8± 5.6	44.8± 40.3	1.6± 1.8	28.2± 22.6	7.1± 0.6	377.5 ± 25.0	5
LHA	-	9.5	904	-	2	39	-	-	9	384	1
LHA	-	-	-	830	-		-	34.1		7.7	2
LHA	21.23	7.54	450.1	-	-	-	-	1.12	12.8		3
LHO	23.8± 2.5	8.5± 0.4	1369.8 ±544.2	1813.7 ±714.7	2.2±1. 8	22.2± 11.5	8.4± 4.8	24.1± 3.9	2.2± 2.2	556.7 ±126. 0	5
LHO		9	1602		12	48			0.8	820	1
LBA	24.2± 2.1	8.4± 0.3	593.1± 63.5	845.5± 92.3	3.2±0. 8	18.8± 7.5	3.7± 5.0	20.7± 5.9	2.7± 4.0	465.0 ±91.2	5
LTA	18.6± 1.1	6.6± 0.3	255.9± 332.9	356.3± 443.7	3.20± 1.30	2.38± 0.16	0.38 ±0.3 1	4.95± 1.49	0.24 ±0.0 91	74.00 ±7.42	5
LTA	-	8.4	103	-	0.2	1.6	-	-	-	92.7	1
LN	-	7.1 ± 0.0	14.9 ± 2.5	27.9 ± 0.7,	-	2.3 ± 0.1	0.2 ± 0.0	0.02 ± 0.0	-	29.3 ± 0.8	4

*[a] Data reported in mg/L except for pH, EC (mS/cm) and Temperature(^OC); * LHA, Lake Hawassa (Ethiopia); LHO, Lake Hora Arsedie (Ethiopia); LBA, Lake Babogaya (Ethiopia); LTA, Lake Tana (Ethiopia);LN, Lake Nabugabo (Uganda); ** 1= Tenalem,(2009);2= Eva et al.,(2011);3= Admasu et al., (2015); 4=Nakirya, (2015);5=This study*

Except Lake Tana, the average Alkalinity level of LHO, LBA and LHA was greater than 377mg/L which is greater than what was reported 301.8mg/L from Indian Pandu Lake (Venkata, 2010). Natural waters may contain a variety of dissolved alkaline substances such as carbonates, bicarbonates, hydroxides and to less extent borates, phosphates and silicates. At neutral pH the alkalinity drives mainly from the presence of bicarbonates. In our study none of the sampled lake water had neutral pH and the alkalinity nature comes other than the carbonates.

The mean Phosphate from the Lakes was greater than 20mg/L except that of Lake Tana (LTA), Phosphate may play its own part for the level of alkalinity. Alkalinity content of a water helps to determine the aggressiveness or scale forming tendency of the water .So control of alkalinity important part of water treatment program.

Water quality is strongly influenced by human factors in combination with the geology, warm climate, and physiographic factors such as rugged terrain, through effects on rates of mineralization, soil erosion and transport of particulates and solutes (Zinabu *et al*., 2002). Since as new *Legionella spp.,* are indentified from time to time from different contintens of aquatic environment, there might be a room for discovery of new *Legionella Spp.,* from African water environments as more survillance of water conducted. So detail study has to be conducted on *Legionella* related issues in Africa.

Even though information is lacking about the water quality guideline value for freshwater systems in relation to risk of transmission of pathogens through recreational use, certain groups of a society like the young, elderly and immunocompromized people visiting the area are susceptible for certain diseases (WHO,2003).

CHAPTER FIVE

5. CONCLUSION AND FUTURE PERSPECTIVES

5.1. Conclusions

In this study *Legionella* species have been isolated 43% (hot springs), 38.6% (hospitals water system), 30.7% (Addis Ababa water distribution system) and 61.3% (natural water bodies). Except with *HPC*, Free residual chlorine, *Legionella* prevalence has weak correlational associations with the measured physicochemical water quality parameters. But the analyzed water samples had Temperature (18.6-48.3^{O}C); pH (6.6-8.5); Sulfate (2.2-59.7mg/L); Phosphate (4.95-48.3mg/L); Potassium (1.9-44.8mg/L); Iron (0.34-23.1mg/L); Fluoride (0.21-30.4mg/L); TDS (88.7-2569mg/L). *Legionella* and *HPC* were present form Log_{10} 1.22-3.22CFU/L Log_{10} 2.1-3.79C CFU/mL respectively. This study is unique in that it indicates the presence of *Legionella* in Ethiopian water sources that has diverse physicochemical parameters for the first time.

In line with the continual growth of urbanization, susceptible human population groups (the elderly, immunosuppressed/immunocompromized people) in Ethiopia, people might have been infected with emerging waterborne pathogens like *Legionella* from hospitals, hot springs and others water distribution system. Since hospitals are giving service to highly vulnerable people, the water distribution system of their complex buildings has to be properly managed with reasonable steps/standards for opportunistic infections (*Legionella*) regardless of the treatment regime and disinfection type employed by the water supplier.

Biofilms in water distribution system are reservoirs for different types of microbes including waterborne emerging pathogens. The presence of relatively higher level of *HPC* in water distribution system indicates the presence of biofilms. Biofilm control plan is crucial for prevention and remediation for biofilm related problems in water distribution lines. Systems that maintain an adequate treatment residual and practice good pipe maintenance will have a lower risk of developing a biofilm problem. Low level of residual free Chlorine(<0.2mg/L) from water samples from Addis Ababa water (AAWDS) and hospitals water distribution system indicates lack of safe potable water supply, effective management and operation throughout the water-supply chain system. The proportion of human population exposed to poorly managed waster systems is increasing and for microbial safety of water in distribution system, periodic assessment of *heterotrophic plate count (HPC),* thermotolerant coliforms, *E. coli*, *Pseudomonas aeruginosa, Legionella spp*. and *Staphylococcus aureus* are important but this is not usually observed in Ethiopia.

Hot springs are reported as disease healing, recreational centers. But microbial infections (E.g. *Legionella*) from hot springs are also reported. To reduce the possible microbial risk, recreational water safety plan for hot spring facilities needs attention by owners, stakeholders and regulatory bodies. Even though, information is scarce about the water quality guideline value for freshwater systems in relation to risk of transmission of pathogens through recreational use, certain groups of a population like the young, elderly and immunocompromized people visiting the area are susceptible for certain diseases.

The present study can enlarge our knowledge towards *Legionella* prevalence and associated physical chemical and microbial parameters from Ethiopian water.

5.2. Future Perspectives /Recommendations

- This is a preliminary research reporting the presence of *Legionella* from different water systems in Ethiopia. Culture method used in this study may underestimate the presence of *Legionella* in water systems due to the viable but not cultivable (VBNC) state of the species, so culture-based standard techniques together with more advanced molecular level of species detections and epidemiological studies are highly recommended for further study. The diversity and potentiality of *Legionella* species to cause disease to humans need to be determined by further study at molecular level.

- Hot Springs are potential sites for ecotourism and are expanding in Ethiopia. Hot spring development should take in to account the possible risk of microbial, chemical and physical health impact to humans and recreational water safety plan for hot spring facilities needs attention by owners, stakeholders and regulatory bodies.

- Emphasis has to be given potable water systems that maintain an adequate treatment residual and practice good pipe maintenance especially in hospitals that has complex buildings and water distribution system.

- Hot springs and natural water bodies should be closely monitored and from public health point of view microbial safety survey, control and prevention mechanisms are needed.

- Education and awareness has to be given for potable water suppliers, property or building owners about risks associated with *Legionella* and others infections.

- Potable water supplier has to maintain standardize water quality parameters. Institutions have to play their own part in managing their building water system.

Owners, managers has to give emphasis to manage their water supply system for controlling microbial risks regardless of the treatment regimen and disinfectant type employed by the public water supplier

- To manage the risk of infections from microbes in water, periodic inclusive microbial test from water distribution system is necessary.

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