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Coliforms as an indicator of waterborne microbial pathogens

– Identification using DNA sequencing

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Abstract

Coliforms are a group of bacteria that since the end of the 1900s' have been used as an indicator for fecal contamination of water. With advances in microbiology in the 1900's it became clear that the coliform group is large and varied group of bacteria and not ideal to use as indicator of waterborne pathogens. However, in lack of better indicator organisms for fecal pollution of water, it is still used. The aims of this study are to give an overview of water quality in the view of global goals, to identify coliform bacteria from water samples and to discuss the results in the light of new achievements in ongoing development of new identification methods. In this study coliforms from collected water samples have been identified and their presumed identity based on growth on chromogenic differential agar have been compared with 16S rDNA-sequencing. Out of 14 samples, 12 where confirmed to be coliforms by the 16S rDNA-sequencing. In addition to identify bacteria from water samples the different detection methods of coliforms are discussed. In relationship to the global goals, which aims to achieve universal access to safe and affordable drinking water by 2030, it is concluded that new, faster and cheaper methods are required to achieve universal basic WASH services by 2030. A suggestion on such a method is Loop-Mediated Isothermal Amplification (LAMP).

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Background

International developing goals and water safety on a global level

Before the turn of the millennium the 191 member countries of the UN agreed on eight Millennium Development goals (MDG) which were aimed to be achieved by the year 2015 (WHO, 2019). The goals aimed to combat poverty, hunger, disease, child mortality, illiteracy and ensure environmental sustainability as well as promote gender equality and empower women. In 2015 the MDGs were replaced by the Sustainable Development Goals (SDG; UN, 2019). The SDGs' were agreed upon by the member States of the United Nations when they adopted the 2030 Agenda for Sustainable Development (OMS and UNICEF, 2017). The 2030 Agenda consists of 17 sustainable development goals and 169 targets addressing developing questions regarding economic, environmental and social issues. The goals seek to end poverty, protect the planet and ensure prosperity for all. The SDGs are global targets and intended to be universally relevant. Both the MDG and SDG contains specific goals targeting water safety, which is a necessity for human life.

Since 1990 the WHO/UNICEF Joint Monitoring Program (JMP) has been producing regular estimates of global progress on drinking water, sanitation and hygiene (WASH; OMS and UNICEF, 2017). JMP was responsible for monitoring the 2015 Millennium Development Goals (MDG) target 7c, which aimed to halve the proportion of the global population without adequate access to water and sanitation by the year 2015. When evaluating the MDG target 7c, it was seen that the proportion of the population with at least basic drinking water services had increased by an average of 0.49 percentage points per year between 2000 and 2015. Notably, the increase was significantly faster in sub-Saharan Africa (0.8) and Eastern- and South-eastern Asia (0.97). Australia, Europe, New Zeeland and North America are according to UNICEF (2017) very close to achieving universal basic drinking water services (OMS and UNICEF, 2017). Latin America, the Caribbean and East and Southeast Asia are on track to achieve universal access by 2030. In 2015 89% of the global population used at least basic drinking water services

The JMP is now responsible for tracking the progress of the 2030 Sustainable Development Goals (SDG) related to WASH (OMS and UNICEF 2017). The targets focusing on improving the standard of WASH services are all under SDG 6 (Box. 1).

UNICEF (2017) states that "The 844 million people who still lacked a basic drinking water service in 2015 either used improved sources with water collection times exceeding 30 minutes (limited services), unprotected wells and springs (unimproved sources), or

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Box 1

GOAL 6. Ensure availability and sustainable management of water and sanitation for all

6.1 By 2030, achieve universal and equitable access to safe and affordable drinking water for all

6.2 By 2030, achieve access to adequate and equitable sanitation and hygiene for all and end open defecation, paying special attention to the needs of women and girls and those in vulnerable situations

6.3 By 2030, improve water quality by reducing pollution, eliminating dumping and minimizing release of hazardous chemicals and materials, halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally

6.4 By 2030, substantially increase water-use efficiency across all sectors and ensure sustainable withdrawals and supply of freshwater to address water scarcity and substantially reduce the number of people suffering from water scarcity

6.5 By 2030, implement integrated water resources management at all levels, including through transboundary cooperation as appropriate

6.6 By 2020, protect and restore water-related ecosystems, including mountains, forests, wetlands, rivers, aquifers and lakes

6.a By 2030, expand international cooperation and capacity-building support to developing countries in water- and sanitation-related activities and programmes, including water harvesting, desalination, water efficiency, wastewater treatment, recycling and reuse technologies

6.b Support and strengthen the participation of local communities in improving water and sanitation management

Box 1, The sixth UN sustainable development goal (WHO and UNICEF, 2017)

took water directly from surface water sources" (WHO and UNICEF, 2017). Previous analysis done by JMP have shown that water collection from unimproved sources is more likely to take over 30 minutes and in 8 out of 10 households' women and girls are responsible for water collection. This creates a double burden for an already exposed population. UNICEF (2017) further states that while billions of people have gained access to basic services since the year 2000, faster progress is required to achieve basic WASH services by 2030. Today there are major gaps in data and to achieve the 2030-goals significant improvements in the availability and quality of data underpinning national, regional and approval estimates of progress are required.

Definition of water safety

According to WHO, safely managed drinking water is defined as an improved drinking water source that is located on premises, meaning a house or building together with its land and outbuildings, available when needed and free from fecal and priority chemical contamination (WHO and UNICEF, 2017). In terms of water safety, the highest priority for global monitoring are fecal, arsenic and fluoride contamination. Arsenic and fluoride contamination are more of a problem in certain parts of the world whereas fecal microbial contamination is a universal concern. To measure fecal contamination *Escherichia coli* or thermotolerant coliforms are used as indicators.

Objectives

The objective of this study was to: (1) Give an overview of water quality in the view of global goals, (2) identify coliform bacteria from water samples, collected from a secondary effluent, and compare its presumptive identity based on growth in chromogenic differential Agar with 16S rRNA-gene sequencing, and (3) to discuss results in the light of new achievements in on-going development of new methods for identification of coliforms.

Introduction

Water treatment

Only 38 % of the global population have access to sewer connections (WHO and UNICEF, 2017). In urban areas the number is 63 % and in rural areas 9 %. If a household has sewer connection, they are classified as having safely managed sanitation services if the toilets are not shared and if the wastes flushed out of the household reach a treatment plant and undergo several steps of treatment. An effluent is the outflow of water to a natural body of water or from a structure such as a wastewater treatment plant, sewer pipe or industrial outfall (Folger, 2016). According to the United States Environmental Protection Agency (US EPA), an effluent is "Wastewater, treated or untreated, that flows out of a treatment plant, sewer, or industrial outfall. This term generally refers to wastes discharged into surface waters". Effluent that has been treated are sometimes referred to as secondary effluent (Laws et al., 2018).

The wastewater treatment process can be divided in the four steps; preliminary treatment, primary treatment, secondary treatment and tertiary and/or advanced treatment (Pescod, 1992). The main objective of the preliminary treatment is to remove coarse solids and other

large materials found in raw wastewater. The primary treatment step is a mechanical, physical or chemical process involving settlement of suspended solids to reduce the total suspended solids of the incoming water by at least 50 % and biochemical oxygen demand (BOD) with at least 20 % before discharge. The BOD is the dissolved oxygen needed by aerobic bacteria to break down organic material present in the water sample. The following step is called secondary treatment and generally involves biological or other treatment with secondary settlement to reduce the BOD further. This step also lowers the chemical oxygen demand (COD) with at least 75%. The COD is an indicative measurement of how much oxygen that is consumed by chemical reactions in when organic matter is degraded, it thereby indicates how much organics there is in a water sample. The last step of the treatment procedure is the tertiary and/or advanced treatment and are aimed to remove nitrogen, phosphorus or any other pollutant, for example microbiological pollution.

International regulations

In 1958 The World Health Organization (WHO) published the first edition of their Guidelines for Drinking-Water Quality (GDWQ; WHO, 2017). The 4th edition of the guidelines, which is the latest version, were issued in 2017. The GDWQ includes recommendations for inorganic, organic and microbial parameters as well as other parameters relating to acceptability such as taste, odor and appearance for drinking water. According to WHO (2017)*,* the GDWQ are "an international reference point of the establishment of national or regional regulations and standards for water safety". WHO (2017) also states that verification of microbial water safety normally is based on testing of indicator organisms. GDWQ advises that the presence of *E. coli* or thermotolerant coliforms provides evidence of recent faecal contamination and therefor have a Guideline Value (GV) saying that no thermotolerant coliforms can be detected in 100 ml samples if the water is to be classified as safe drinking water. However, WHO does not have a GV for the presence of coliforms in drinking water samples.

The United Nations (UN) refers to the GDQW in the question of safe drinking water: "The water required for each personal or domestic use must be safe, therefore free from micro-organisms, chemical substances and radiological hazards that constitute a threat to a person's health. Measures of drinking-water safety are usually defined by national and/or local standards for drinking-water quality. The World Health Organization (WHO) Guidelines for Drinking-Water Quality provide a basis for the development of national standards that, if properly implemented, will ensure the safety of drinking-water" (UN, 2014)*.*

National regulations

In the United States the US EPA sets regulatory limits for the amounts of certain contaminants in water (EPA, 2017). They also identify contaminants in drinking water to protect public health. The contaminant standards that EPA works with are required by the "Safe Drinking Water Act" which is the main federal law that ensures the quality of Americans' drinking water. The law authorizes EPA to set national
standards for drinking water to protect against health effects from exposure to naturally occurring and man-made contaminants. EPA uses Total Coliforms as one of their indicator organisms

In Sweden regulations, recommendations and communication regarding secure food and drinking water is authorized by the Swedish National Food Agency (The Swedish Food Agency, 2019) . The food legalizations in Sweden are essentially harmonized with the EU,

and Swedish National Food Agency takes an active part in the development of new legislation in co-operation with other EU member states. In addition to the EU regulation the Swedish Food act, the Swedish Food Regulation and Swedish National Food Agency give recommendations regarding drinking water, The Swedish Food Regulation, (LIVSFS 2017:2) states that no *E. coli* should be detected in a 100 ml sample for the water to be classified as safe (Livsmedelsverket, 2017). However, up to 10 total coliforms can be detected in a 100 ml sample for the water to be classified as safe.

The growth of Coliform concept

The term coliform or coli-aerogenes, was first used in the late 1800s' by British bacteriologists to describe bacteria in fecally contaminated water (Leclerc et al, 2001). American scientists later defined coliforms as "lactose fermenting, Gram-negative bacteria used for the detection of water pollution" (Box 2). With time more bacteria that fitted the description of a coliform were discovered and the group of "lactose fermenting, Gram-negative bacteria used for the detection of water pollution" have also been called "the colon group", "Escherichia-Aerobacter group" and "Coli-Aerogenes group" (Leclerc et al., 2001). The oldest members of the traditional coliform group are *Klebsiella pneumoniae*, *K. rhinocleromatis*, *Bacterium coli* (later called *E. coli*), *Bacterium lactic aerogenes* (later *Enterobacter aerigenes*), *Bacillus clocae* (later *Enterobacter cloacae*) and *Bacterium freundii* (later *Citrobacter freundi*).

| Box 2. |
| **Definitions of coliform bacteria** |
| |
| **Coliforms** - Large and varied group of bacteria, first used to describe bacteria in fecally contaminated water. Coliforms are lactose fermenting and growing at 35°-37° C. |
| |
| **Thermotolerant coliforms or Fecal Coliforms**- Coliforms with the capability to grow at 44° - 45° C. |
| |
| ***Escherichia coli* (E.coli)** - The most common Gram-negative, facultative anaerobic bacteria found in feces. Is part of the coliform group. |

Box 2, Definitions of coliform bacteria

In 1892 *E. coli* was suggested as an indicator in water monitoring (Leclerc et al., 2001). *E.coli* is named after the German-Austrian pediatrician Theodor Escherish (Etymologica, 2015). Escherish used anaerobic culture methods and Hands Christian Gram's new staining techinique, gram staining, when he isolated a variety of bacteria from infant fecal samples in 1885. He called the Gram-negative, facultatively anaerobic bacteria he found *Bacterium coli*. In 1958 the *Bacterium coli* officially got recognized as *Escherichia coli*.

During the early 20-th century, when biochemical approaches were discovered to define bacteria, it became clear that the old definition of coliforms as a group was too involved for practical application (Leclerc et al., 2001). A second definition of the coliform group was defined with the so called IMViC-test(Parr, 1936). The IMViC test is a set of four biochemical tests, where I stands for indole test (indol production) , M for methyl red test (production of acids from glucose), V for Voges–Proskauers test (production of 2,3 butane diole) and C for citrate test (capacity to use citrate as the sole source of carbon). This definition meant the coliforms fermented sucrose and dulcitol as well as they produced indole and an acetyl methyl. This specific test battery meant that some bacteria that originally belonged to the coliform group now were excluded from the group. Those excluded were called "atypical coliforms" and are divided into two classes. The first group included bacteria existing in fecal contaminated water that tested positive on most of the tests in the IMViC battery but differed to a slight degree. For example, *E. coli* belongs to this group. In the

second group of "atypical coliforms" were those who normally were found in fecal contaminated water but who's reaction to lactose differed from the coliforms that fermented lactose.

During the 1960 great genomic founding were made which lead to an improvement in the differentiation of *Enterobacteriaceae* and coliforms (Leclerc et al., 2001). The old taxonomic approaches to classify bacteria based on biochemical techniques was challenged by genomic approaches. New recommendations said that genomic species should encompass 70% or greater DNA-DNA relatedness with 5°C or less ΔTm (change in DNA melting temperature). However, what was called the coliform group, with the traditional classification, was now defined as bacteria testing positive on an o-Nitrophenyl-β-D-galactopyranoside test (ONPG-test) together with the family *Enterobacteriaceae*. That a bacteria tests positive on an ONPG test means that it has the enzyme β-galactosidase.

The high degree of variation in the so called "original" coliform group led to a conclusion that the coliform group was a large group of microorganisms all members of the *Enterobacteriaceae* family with lactose fermentation capabilities but not all members of this family are coliform (Leclerc et al., 2001). The term coliform is largely determined by the US EPA approved method capability to discriminate between genera. Thus, this method does not distinguish its pathogenesis or phylogenetic components. Figure 1 shows the phylogenetic distance between true coliforms and closely related organisms that aren't coliforms as defined by current EPA approved methods.

In 1996 the WHO defined coliforms to be "Gram-negative, rod-shaped
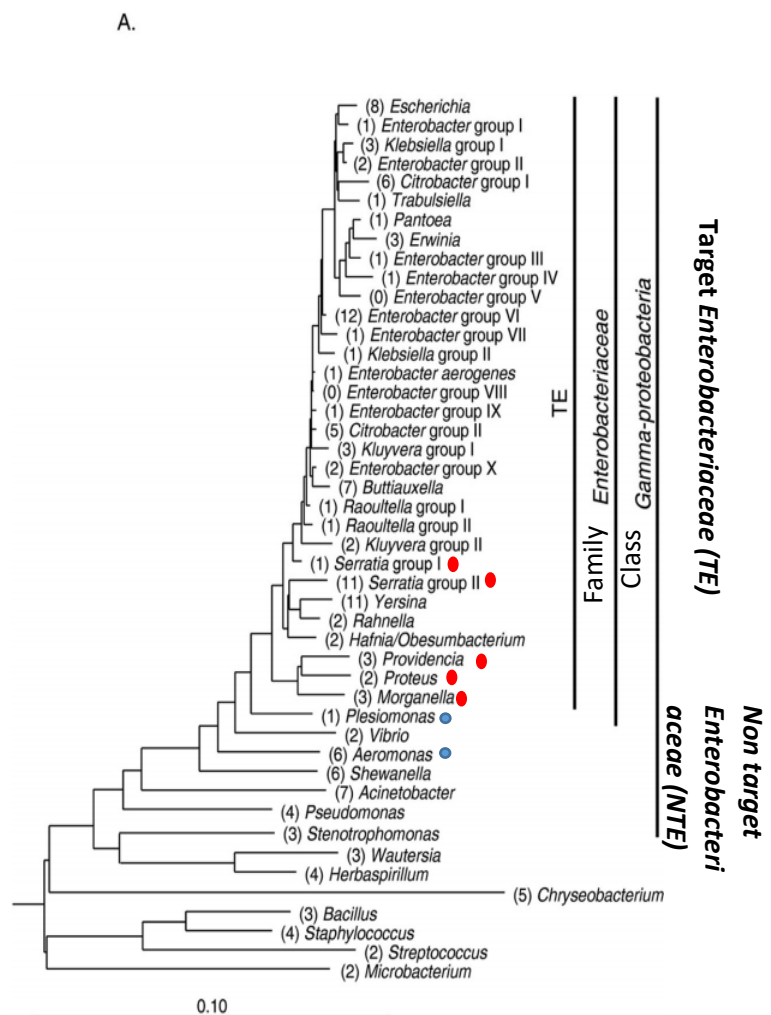
Figure 1, Phylogenetic analysis of coliform classification with particular attention no-coliforms members of the family Enterobacteriaceae and class gamma-proteobacteria. These coliforms and related species 16s rDNA phylogenetic analysis were modified from (Zhang et al., 2015). Red dots are organism that often show false positive and blue dots false negatives on EPA approved methods due to its genetic relationship to target coliforms.

bacteria, capable to grow in the presence of bile salts or other surface-active agents with similar growth inhibiting properties, and able to ferment lactose at 35-37ºC with the production of acid, gas and aldehyde within 24-48 hours" (Leclerc et al., 2001). The definition also included the requirement of the bacteria to be oxidase negative, non-spore forming and display β-galactosidase activity.

Indicator organisms

In general, an indicator organism is an organism whose presence indicates the condition of a substance or environment, for example the potential presence of pathogens. Traditionally the term coliforms have been used as indicator of fecal pollution. However, as a consequence of the advances in molecular genetics and better understanding of the *Enterobacteriaceae* family classification, WHO are no longer using the term coliform as a unison term for indicator organisms in water. Instead they use the groups *Enterobacteriaceae* and thermotolerant coliform as indicator organisms (Kim et al., 2016) (Woolverton, Sheerwood, & Woolverton, 2014).

The human microbiota contains of more than 500 different strains and new data are continually obtained (Leclerc et al., 2001). The bacteria isolated from the lumen are predominately anaerobic and facultative. Many of the bacteria in the human microbiota are difficult to cultivate due that they have poor survival skills outside of the lumen. Coliforms are the most common Gram-negative, facultative anaerobes in feces making them suitable to use as indicator organisms. Compared to other microorganism of the gut microbiota they are also easier to cultivate and have the skills to survive outside of the lumen. *E. coli* are the most common of the coliforms found in feces since it is a permanent member of the gut microbiota. Today 147 different bacteria strains are included in the coliform group (Maheux et al., 2014)

Coliforms or *E.coli* are though being useful and valuable not an ideal indicator organisms (Leclerc et al., 2001). The traditional microbial tests for coliforms may give false positives since the coliform group is so diverse, the test might therefor give a positive result for a bacterium that is not a coliform. Traditional tests might also give false negatives since pathogenic fecal bacteria might not be detected (Leclers et al. 2001). A reason for this can be that some fecal bacteria are strict anaerobes and therefor difficult to cultivate. Research is still going on to find the "ideal" indicator organisms to use in sanitary microbiology. Criteria for such an "ideal" indicator organism can be seen in Fig. 2 (Woolverton et al., 2014).
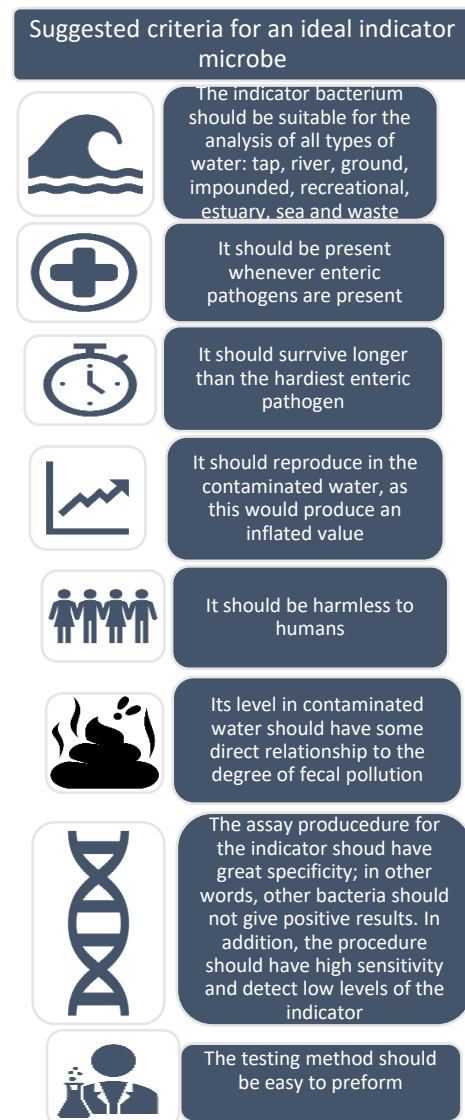
Suggested criteria for an ideal indicator microbe
The indicator bacterium should be suitable for the analysis of all types of water: tap, river, ground, impounded, recreational, estuary, sea and waste
It should be present whenever enteric pathogens are present
It should surrvive longer than the hardiest enteric pathogen
It should reproduce in the contaminated water, as this would produce an inflated value
It should be harmless to humans
Its level in contaminated water should have some direct relationship to the degree of fecal pollution
The assay producedure for the indicator shoud have great specificity; in other words, other bacteria should not give positive results. In addition, the procedure should have high sensitivity and detect low levels of the indicator
The testing method should be easy to preform

Figure 2, Suggested criteria for an ideal indicator microbe (Woolverton et al., 2014)

Methods for detection of total coliforms and Escherichia coli

Since the definition of coliforms are based on metabolic and growth dependent methods these are still the main methods used to identify coliforms. The methods referred to as traditional methods include the multiple-tube fermentation technique and the membrane filter technique (Rompre, Servais, Baudart, De-Roubin, & Laurent, 2002). The multiple tube fermentation technique includes a primary and a confirmatory test. The primary test includes a serial of decimal dilutions of the water sample inoculated in lactose or lauryl tryptose broth. Production of gas after 48 hours of incubation at 35°C indicates a positive test. The conformation test uses brilliant green lactose bile broth, gas formation within 48 hours of incubation at 35°C indicates a positive result and thereby presence of coliforms. The confirmation test can also use an EC medium (buffered lactose broth with bile salts inhibiting Gram positive cocci and spore formers) for 24h at 44.5°C to detect fecal thermotolerant coliforms.

In the membrane filter technique the water samples are filtered through a sterile filter which is then incubated on a selective media (Rompre et al., 2002). This medium can for example be m-Endotype media, Tergitol-TCC medium, MacConkey or Teepol media (Rompre et al., 2002). The filter is incubated on top of a plate with media at 35°C for 24 h and a color differention of the bacteria colonies formed indicates the presence of coliform. To detect Fecal Coliforms a lactose enriched media can be used, the incubation time is then 24 h at 44.5°C.

With the invention of polymerase chain reaction (PCR) it became possible to identify bacteria based on conserved regions in their DNA. Matheux et al. (2014) compared results from PCR, targeting the lacZ-gene, wecG-gene and 16S rRNA-gene, with the results of traditional culture-based methods (Maheux et al., 2014). This was done to measure the ability of the different methods to detect coliforms in potable water. The results showed that coliforms in water samples ca be detected using molecular assays, even if the coliform strain is highly genetically variable. It was also shown that the 16S rRNA-gene was a more efficient target, compared to the lacZ and wecG genes. It was concluded that PCR based on 16S rRNA-gene sequencing is as efficient as culture-based methods in the detection of coliforms.

Since culture-based methods simply rely on the activity of one single gene these methods can give false positive or negative results when detecting coliforms in water samples (Maheux et al., 2014). For example, medium that contains lactose and are confirmed positive by gas production relies on the bacterium gene expression of β-galactosidase. According to Matheux et al (2014) molecular methods are more reliable but due to that some bacterial species preform genetic polymorphism, these methods can also result in false negative or positive results. Zhang et al. (2015) concluded that it was most effective to combine results from molecular methods and culture-based methods when detecting coliform

Loop-Mediated Isothermal Amplification (LAMP) is a molecular method that also can be used to identify bacteria in potable water samples (Tanner & Evans, 2014). It works similar to PCR in the way that a reaction mixture with buffer, primers, dNTS are used. However, it does not require thermal cycling equipment and is typically fast and less prone to inhibition from environmental samples. The equipment required for preforming LAMP also has portability potential and is inexpensive which gives the technique great potential in the future.

Methods

Water collection and coliform isolation

A water samples of 4 L were collected from a secondary effluent in Maryland, USA, during February 2019. Transport was done at ambient temperature immediately after sampling and refrigerated upon arrival to the lab. The water sample were plated to 14 ECC ChromoSelect Agar (ECC; GRG International Inc., Springfield, USA) spreading 100 µL of water on each plate. The plates were then incubated for 24 h at 37°C. Presumptive coliforms were selected based on color (pink indicated coliforms according to specification to the medium) and transferred to Lauryl Tryptose Agar (LTA; VWR International, Atlanta, USA).

The isolated colonies were stored in a fridge (4°C) and later used for colony PCR (cPCR). The day before preforming the cPCR the selected colony was transferred to 10 mL of Liquid Tryptose Broth Medium (LTB; VWR International, Atlanta, USA) and incubated for 16 to 18 h in a 37°C.

After the incubation the concentration of nucleic acid in the samples was measured with a Nano Drop™, 2000C Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Stock preparation

The concentration of bacteria after incubation in LTB was estimated by measuring the optical density (OD600) with a spectrophotometer (Beckman Coulter DU(R) 530 Life Science UV/Vis, Boston, MA, USA). Stocks of the microorganisms were prepared by taking 90 mL of culture (grown in LTB) and 100 mL of glycerol in test tubes. The stock-samples were sealed properly with a plastic lid and stored in a -80oC freezer.

Colony PCR (cPCR) and gel electrophoresis

The 16R cPCR amplification was performed according to specifications using OneTaq® 2X Master Mix (New England Biolabs Inc, MA, USA) with Standard Buffer, 16S rDNA bacterial universal primers (27 Forward and 1492 Reverse; Integrated DNA Technologies, Iowa, USA; Weisburg, Barns, Pelletier and Lane, 1991)), bacteria sample and sterile water for a total reaction volume of 50 µL. The reaction was performed in a MJ Research PTC-200 PCR thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). The reaction was carried out using the following protocol: 35 cycles of 96°C for 60 seconds, 50°C for 40 seconds and 72°C for 45 seconds. The cPCR product length and quality were assessed by electrophoresis with samples running for 1 h at 100 V on a 1% agarose gel stained with a fluroces containing GelRed® (Nucleic Acid Gel Stain, Biotium Inc. Hollywood, CA, USA).

Amplification product relative size was determined visually using the GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA). 16s cPCR product fragment (Fig. 2) of ~1.5 kbp were purified using the DNA Clean and Concentrator™-5 (Zymo Research, Irvine, CA, USA) for a final 25 µL of elution volume. Purified cPCR product concentration and quality were measured using NanoDrop™ 2000c Spectrophotometer. Samples of 300-350 ng were prepared for Sanger sequencing as instructed by the Cornell sequencing facility using the following primers, 27F and 1492R.

The sequencing result were treated using Benchling software and analyzed using NCBI BLAST® (National Information Center for Biotechnology, 2019).

Results

The results from the gel electrophoresis ran after the 16S rDNA cPCR showed that DNA had been amplified in all the 14 samples (Fig. 3). The expected 16S rDNA product size using the primer setup was 1.5 kbp which is consistent with figure bands in well 1-14. Well 15 contained a no-template control (NTC) and showed no band.

The NCBI BLAST tool affiliated the 14 isolates sequenced to the genera *Klebsiella, Enterobacter* or *Aeromonas* (Table 1). Out of the 14 samples, nine were identified as *Klebsiella*, three as *Enterobacter* and two as *Aeromonas*. All of the samples displayed identity score over 97% (Table 1). Based on the current bacteria identification convention identity score >97% is enough to determine if an organism (query sequence) belong to the same group as the best match in the database. The samples query coverage ranged from 98-100%. Query coverage is a comparison of the size of the sequences, a higher percentage refer to similar size. Queries with identity percentages of <97% are consider member of the same family and likely the same genera.
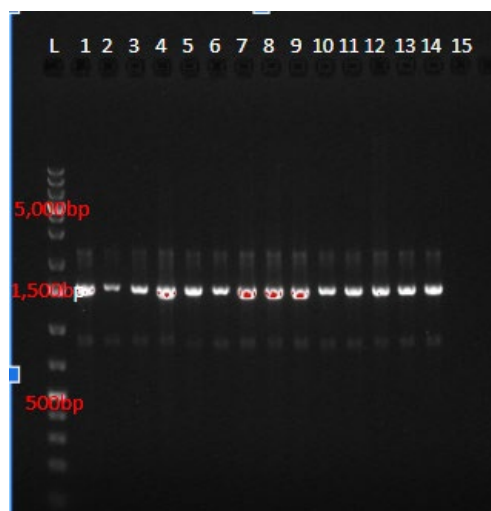

Figure 3, Results from gel electrophoresis of the 15 samples. Wells numbers corresponds to sample number in the study. L stands for ladder which was 1 kb plus. Well 15 is a negative control.

Discussion

Fourteen strains of coliforms were isolated from secondary effluent using cultivation on ECC ChromoSelect Agar. From the results of the gel electrophoresis it can be seen that the cPCR succeeded to pick up band sizes (1.5 kbp.) expected from different coliform groups as the DNA, targeted with the primers, had been multiplied in all 14 bacterial isolates originating from secondary wastewater effluent. The sequencing result showed that the isolated bacteria either belonged to the genera *Klebsiella, Enterobacter* or *Aeromonas*. *Klebsiella* are Gram negative and non-motile, it belongs to the family *Enterobacteriacae* and is a coliform(World

Table 1, Sequencing result with query cover and presumed identity from NCBI BLAST®

Sample	Presumed Identity	Query cover (%)	Per. Ident (%)
1	*Klebsiella pneumoniae*	100	100.0
2	*Enterobacter sp. strain*	100	99.38
3	*Enterobacter sp. strain*	99	97.91
4	*Enterobacter sp. strain*	98	98.73
5	*Klebsiella pneumoniae*	99	100
6	*Klebsiella pneumoniae*	100	98.97
7	*Klebsiella pneumoniae*	98	97.53
8	*Klebsiella pneumoniae*	100	97.37
9	*Klebsiella sp.strain*	99	97.21
10	*Aeromonas hydrophila*	100	99.95
11	*Aeromonas caviae*	99	97.25
12	*Klebsiella pneumoniae*	100	98.86
13	*Klebsiella pneumoniae*	98	97.59
14	*Klebsiella pneumoniae strain NJ8 16S*	99	98.96

Health organisation, 2011). *Enterobacter* is biochemical similar to *Klebsiella* and is also a coliform. Several of *Enterobacter* and *Klebsiella* strains are opportunistic pathogens that can cause urinary and respiratory infections. *Aeromonas* resembles the members of the *Entereobactericae* but are not 'coliforms (Janda and Abbott, 2010). *Aeromonas* are disease-causing pathogen of fish and other cold-blooded species and an etiologic agent responsible for a variety of infectious complications.

Klebsiella and *Enterobacter* are both part of the coliform groups while *Aeromonas* are not (Maheux et al., 2014). Since samples were selected based on the assumption that they were coliforms by the traditional cultivation-based methods the sequencing result shows that the culture-based method did not match the sequencing result in all the samples. However, 12 out of the 14 samples, were as the culture-based method indicated, confirmed to be coliforms. This indicates that the secondary effluent, indeed, were contaminated with fecal bacteria. This is not surprising as, in general, wastewater treatment methods for cleaning municipal wastewater are designed for removing organic matter and nutrients like nitrogen and phosphorus, paying little attention to the pathogens.

Even though all of the samples had a query score over 97% further bioinformatic strategies are necessary to confirm its' identity with more accuracy(National Information Center for Biotechnology, 2019). It might also be necessary to test more than 14 water samples. In this case with more time and funding the samples could be rework with a paid software with a proprietary algorithm for cleaning and aligning consensus Sanger sequences. In addition to sequence the same template with internal primers to improve resolution of the 1.5 kbp sequence.

It can be discussed if using the group of coliforms as an indicator organism is accurate today (Leclerc et al., 2001). When the term coliforms first was used it was with the attempt to describe bacteria in fecally contaminated water. Already in the early 1900s it became clear that the definition of a coliform is too broad for practical detection of waterborne pathogens. With the discovery of molecular genetic methods, the old taxonomic system was challenged. If a bacterium is a coliform or not does not have any anchoring in genetics and can therefore be seen as outdated.

Using PCR in detecting waterborne pathogens can be inconvenient since it is laborious and consumes resources to perform. The equipment and recourses PCR require might not be available in low income countries without basic WASH services (Kim et al., 2016). In an emergency situation, such as a natural disaster, it is urgent to know if water effluents are free from fecal contamination. In those situations, the time it takes to isolate and sequence bacteria from the effluent is critical. There is there for a need for new, faster and cheaper methods to detect waterborne pathogens. Since LAMP is an isothermal amplification method it only requires one single temperature, this avoids the need for expensive thermal cycling equipment (Tanner and Evans, 2014). Constant incubation and amplification also do not require temporal constraints from defined cycles. This results in amplification reactions as rapid as 5 minutes. Present achievement in LAMP indicates that this method is a promising alternative technique for detecting coliforms.

The conclusion of this work is that coliforms as a group may be unpractical and not ideal to use as an indicator organism for fecal contamination of water (Leclerc et al., 2001). However, in the lack of other indicator organisms, it is still widely used. The first objective of this study

was to give an overview of water quality in the view of global goals. In relationship to the global goals, which aims to achieve universal access to safe and affordable drinking water by 2030, it can be concluded that faster progress is required to achieve universal basic WASH services by 2030(Kim et al., 2016).

The second objective was to identify coliform bacteria from water samples, collected from a secondary effluent, and compare its presumptive identity based on growth in chromogenic differential Agar with 16S rRNA-gene sequencing. The results showed that the chromogenic media detected some microorganisms to be coliforms which was confirmed not to be coliforms by the 16S rDNA sequencing. However, 12 of 14 samples were confirmed to be coliforms by 16S rRNA-gene sequencing. By using coliform as an indicator organism of fecal contamination it can be concluded that the samples of secondary effluent in the present study was fecally contaminated and, hence, not safe to drink.

The third objective was to discuss results from objective one and two in the light of new achievements in on-going development of new methods for identification of coliforms. In conclusion countries lacking basic WASH services are poorer countries where current equipment for investigating drinking water might not be afforded. Therefor the future requires, new methods to investigate if drinking water is safe accurately. LAMP seems to be a promising method for rapid and cost-effective detection of coliforms.

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