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Subject

A comparative Study of Recent technics used for the detection of *Escherichia coli*, *Legionella* and *Vibrio Cholerae* in water samples.

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Thanks

«Praise to Allah, who has guided us to this; and we would never have been guided if Allah had not guided us»

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Dedication

To our beloved and ever-loving parents for their unconditional support throughout our lives. Thank you for giving the strength to chase our dreams.

To our sisters and brothers , aunties and uncles who deserve our whole hearted thanks too.

To all our friends, thank you for sharing with us the good and bad moments; thank you for your encouragement and support. Your friendship made a wonderful journey of our lives. We cannot list names but you are always in our minds.

To all those who love us.

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Abbreviation list

ATP : Adenosine triphosphate

B.substilis : *Bacillus subtilis*

B-PER : Bactérial protein extraction

CANRY : Cellular analysis and notification of antigen risks and yields

CAP: Community-acquired pneumonia

CARD-FISH: Catalyzed reporter deposition fluorescence in situ hybridization

CFU: Colony forming unit

CRPG : Chlorophenol red-β-d-galactopyranoside

CSR: Class switch recombination

Ct : Cycle threshold

DAG : Diacylglycerol

ddPCR : droplet digital PCR

DFA: Direct fluorescent antibody

DI water : Deionized water

DMF : Dimethylformamide

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

E.coli: *Escherichia coli*

E.faecalis : *Enterococcus faecalis*

EAEC: Enter aggregative *Escherichia coli*

EC : Electric conductivity

EHEC: *Enter hemorrhagic Escherichia coli*

EIEC: Enter invasive *Escherichia coli*

EPA : Enviromental protection agency

EPEC: Enter pathogenic *Escherichia coli*

ETEC: *Enterotoxigenic Escherichia coli*

Eva : Ethylene vinyle Acetate

FA: Fluorescent-antibody

FA-DVC: Fluorescent antibody -direct viable count

FBS :Fetal bovine serum

FISH: Fluorescence in situ hybridization

fura 2-AM : fura 2- acetoxy methyl .

GC-SPR : Controlled grating-coupling surface plasmon resonance

GU : Genomic units

GVPC : Glycine vancomycin polymyxin cycloheximide

HAT : Hypoxanthine aminopterin thymidine .

HBN : Hydroxynaphtol blue

HDA : Helicase-dependent amplification

HGPT : Hypoxanthine-guanine phosphoribosyl transferase

IFAG: Immunofluorescent-aggregation

IFAST : Immiscible filtration assisted by surface tension

IgG : Immunoglobuline G

IgM : Immunoglobuline M

IMS : Immunomagnetic separation

IP3 : Inositol triphosphate

JORA: Official Journal of the Algerian Republic

K_D : K-dimensions

L. spp*: Legionella .spp*

L.pneumophila*: Legionella pneumophila*

LAMP : Loop-mediated isothermal amplification

LB: Lysogeny broth

LD : *Legionnaire's* disease

LF : Lateral flow

LFD: Lateral chromatographic flow dipstick

LOD: Limit of detection

LPS : Lipopolysaccharide

LTB : Lauryl tryptose broth

MAb : Monoclonal antibody

MetGlu: Methyl-beta-D-Glucuronide

MPN : Most probable Number

NASBA : Nucleic acid sequence based amplification

NTC : No template control

OMS : World Health Organisation

PAD: Paper based analytical device

PBS :Phosphate-buffered saline

PCR: Polymerase chain reaction

PIP3 : Phosphatidyl inositol trisphosphate

PLC : Phospholipase C

POC : Point of care

POCT : Point of care tests

RCA : Rolling circle amplification

RDT: rapid diagnostic tests

REG : Resorufin β-D-glucuronide

RFU: Relative fluorescence units

ROX : Carboxy-X- rhodamine

RPA : Recombinase polymerase amplification

RPMI : Roswell park memorial Institute

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

S.enterica : *Salmonella enterica*

SELEX : Systemic evolution of ligands through EXponential enrichment

SEM : Scanning electron microscop

SPR : Surface plasmon resonance

STEC: *Shiga* toxin-producing *Escherichia coli*

SYBR : Sybricons

TCBS :Thiosulfate-citrate-bile salts-sucrose

TEM: Transmission electron microscopy

TMB : Tetramethylbenzidine

UPT-LF: Up-converting phosphor technology-based lateral flow

US EPA : United states enviromental protection agency

V.cholerae : *Vibrio cholerae*

VBNC : Viable but non-culturable

VO1 :*Vibrio* O1

WHO :World health organization

X-Gluc : β-D-glucuronide

UCP : Uncoupling protein

Introduction

Introduction

Water is one of the basic needs and is required by all life on earth and it dominates a majority of the space on our planet , covering about 71% of the total surface area of earth . (Muthu , 2018)

Water consumption and use is defined as the water is drawn continuously from surface or ground and that can be utilized for direct and indirect purposes. Direct purposes include bathing, drinking, and cooking while examples of indirect purposes are theuse of water in processing wood to make paper and in producing steel for automobiles. The bulk of the world's water use is for agriculture, industry, and electricity. (Muthu , 2018)

To protect public health from water contamination especially Microbial Contamination , monitoring microbial water quality is necessary to inform development and implementation of effective water safety management. (Reuter *et al* ., 2019)

Therefore , Researchers have been looking at different methods for easy, rapid, specific and sensitive detection of bacteria in contaminated water samples. It is well known that traditional microbiological testing methods are time consuming, since bacteria have to be isolated, cultured and require a series of biochemical tests for identification and confirmation. In recent years, there have been several approaches that have been pursued towards the development of methods for the detection and quantification of bacteria in water. The conventional approach for the detection and enumeration of bacteria involves filtering the water samples through a membrane filter, followed by counting the number of bacteria colonies of the filtrate sample using the plate counting method.The counted colonies can be related to the number of cells based on which the quality of water is determined. However, these methods take 24 to 48 hours to produce results, often requiring transportation of water samples to a central laboratory and trained personnel to perform the tests. (Reuter *et al* ., 2019)

Alternatively, Several Rapid Detection Methods have been developed using advanced techniques such as : quantum dots ; magnetic beads ; flow cytometry, polymerase chain reaction (PCR) ; microfluidics and lateral flow assays . (Reuter *et al* ., 2019)

In this work , we have analyzed differents studies which demonstrate the recent techniques used for detection of the most common waterborn pathogens such as : *Escherichia*

Coli (*E.coli*) , *Legionella pneumophila (L. pneumophila)* and *Vibrio Cholera (V. Cholerae)* , causing dangerous diseases like *Cholerae* and *legionellosis* .

This work is composed contained 4 chapters:

The first chapter presents a generalities on water.

The second one presents the microbiological characteristics of water.

The third chapter demonstrate the material and methods of these studies.

The fourth chapter reports the results and discussions of each technics used in this studies.

Part 1

Bibliographic part

Chapter1

Generalities on water

1.1 Definition of water

Water is a unique liquid and without it life is impossible. Water plays a vital role in the proper functioning of the earth's ecosystem. Man uses water for various purposes which include drinking, transportation, industrial and domestic use, and irrigation in agriculture recreation, fisheries, and waste disposal among others. Water that is of a good drinking quality is important to human physiology, and man's continued existence depends so much on its availability .The quality of water for drinking deteriorates due to inadequacy of treatment plants, direct discharge of untreated sewage into rivers and stream, and inefficient management of piped water distribution system. The contaminated water therefore has critical impact on all biotic components of the ecosystem and this could affect its use for other purposes. Water receives its bacteria spores from air, sewage, organic waste, dead plants and animal, at times almost all microorganisms may be found in water, but bacteria appeared to be the major water pollutants. Majority of the bacteria found in nature live on dead decaying organic matter as saprophytes. Bacteria also help in the digestion of poisons from food and water. Presence of other species could cause various diseases to man and other animals. Water obtained from wells, boreholes, streams and river are never chemically pure, even rain water contains dissolved materials from the air as well as suspended dust intermixed with microorganisms**.** Impurities in water may be floating as suspended matter consisting of insoluble materials of greater density than water which could be removed by sedimentation and in the form of bacteria. The bacteriological examination of water is performed routinely by microbiologists, and this will ensure a safe supply of water for drinking. bathing, swimming and other domestic and industrial uses.(Adebayo,1999 ; Ajayi et Akonai, 2005; Prescott *et al*., 2008)

Water, an element that can be found in three forms (liquid, gas and solid). The slow and incessant evaporation of rivers, lakes and seas causes the formation in the upper atmosphere of clouds which by condensation transform in rain. A fraction of the rainwater runs off the surface of the soil and will swell the rivers and lakes, where it is subject on the one hand to evaporation on the other hand to seepage through the ground. Part of the seepage water is taken up by the vegetation that it feeds before being released into the atmosphere, it is evapotranspiration. The other part accumulates in the basement to form groundwater tables which, in turn, can form emergent sources at the ground surface (Novotna *et al*., 2019**).**

1.2. Different types of water intended for consumption human

The available reserves of natural water are groundwater (infiltration, groundwater), stagnant surface water (natural or artificial lakes or dams) or currents (river) (Degremont, 2005).

1.2.1. Surface water

Surface water is water that circulates or is stored on the surface of continents. The latter originate either from groundwater which the emergence constitutes a source, namely runoff water (Palamuleni et Akoth ,2015)

They are generally rich in dissolved gases, suspended and organic matter. They are very sensitive to mineral and organic pollution such as nitrates and pesticides (Claude, 2010)

1.2.2. Underground waters

Groundwater is the water in the subsoil that constitutes a supply of water invaluable to mankind. They are traditionally the water resources preferred for drinking water because it is more sheltered from pollution than surface water. The penetration and retention of water in the soil depend on the characteristics of the land in question and in particular their structure which may allow the formation of aquifer resources called aquifers (Guergazi et Achour, 2005)

1.3.Definition of a standard

A standard is a benchmark established in accordance with a regulation or a minimum, average or higher benchmark. It allows you to compare a situation with respect to a threshold value and to define acceptable conditions with respect to one that is not (Hamsatou, 2005)

1.4. Physico-chemical qualities

The physico-chemical quality of the water provides information on the location and evaluation of a pollution level, depending on a set of parameters. Based on values of references, it is assessed using several parameters (Rodier *et al.*, 2009).

1.4.1. Physicochemical characteristics

The sum of the physicochemical properties is summarized in the following Table 1.

Table 1.Drinking water standards according to Algéria **(**OMS , 2000 ; JORA , 2011 **)**

Settings	Unit	Algerian standards	OMS standards
pH	/	6,5 – 8,5	6,5 – 9,2
Temperature	C°	25	-
Conductivity	µs/cm	2800	-
dry residue at180° C	mg / L	2000	1500
Turbidity	NTU	2	5
total hardness	mg / L	500	500
Calcium	mg / L	200	-
Magnesium	mg / L	150	150
Sodium	mg / L	200	-
Potassium	mg / L	20	-
Sulphate	mg / L	400	250
Chlorure	mg / L	500	250
Nitrate	mg / L	50	50
Nitrite	mg / L	0,1	0,1
Aluminum	mg / L	0,2	0,2
Phosphate	mg / L	0,5	0,5
Ammonium	mg / L	0,5	-
organic matter	mg / L	3	-
heavy metals	mg / L	0,3	-
Iron	mg / L	0,3	0,3

WHO : World Health Organisation , **JORA :** Official Journal of the Algerian Republic

Chapter 2

Microbiological Characteristics

Generally, all water resources, whether lakes, rivers, rivers, as well as shallow groundwater, contain 3 types of germs: typically aquatic, telluric (due to runoff) and germs of human or animal contamination (faecal contamination); regardless of the type of germ it can cause infectious diseases in humans (Novotna *et al*.,2019).

Ultimately, the majority of microorganisms come from human or animal excreta, the importance of microbiological pollution requires us to do a treatment before being distributed to the public. The microbiological analysis of water distributed for consumption based on the search for "microorganisms indicating faecal contamination". These indicators are specific to the intestinal flora, they are not necessarily pathogenic, but their presence in large numbers in an aquatic environment indicates the existence of faecal contamination, and therefore a potential epidemiological risk (Kanampalliwar et Singh , 2020).

2.1. Pathogenic bacteria

Pathogenic bacteria act as a warning signal. In fact, only *Salmonella* and *Shigella* are frequently sought after bacteria, apart from cases of epidemics. In recent years, however, some importance has been attributed to *Yersinia*, *Campylobacter, Pseudomonas aeruginosa, Staphylococcus aureus, L.pneumophila , Aeromonas hydrophila, V. Cholerae*. (Palamuleni et Akoth , 2015)

2.1.1 *Vibrionaceae* family

Comprises Gram-negative, motile bacilli (ciliature polar or mixed) or immobile; they are facultative aero-anaerobes, reducing nitrates to nitrites, oxidase (+) and degrading carbohydrates by fermentative metabolism. Four bacterial genera are gathered in this family: *Vibrion* (type genus), *Photobacterium, Plesiomonas* and *Aeromonas* (Mougin *et al* ., 2021)

2.1.1.1. Genus *Vibrio*

The genus *Vibrio* includes more than 30 species, all of which are aquatic habitats; each is strict halophiles, others are not. The most important species of the genus is *Vibrio cholerae*. (Leclerc, 1994).

2.1.1.2. *V. cholerae*

V. cholerae is an autochthonous inhabitant of the aquatic environment such as brackish water and estuaries and exists in association with zoo- and phyto-planktons. Of the over 206

serogroups, only O1 and O139 *V. cholerae* are responsible for causing epidemics and pandemics. *V. cholerae* serogroup O1 is responsible for the majority of cholera cases globally it is now known that *V. cholerae* enters into a viable but non-culturable (VBNC) state under certain environmental conditions, whereby the cells cannot be propagated by conventional culture methods, yet remain capable of producing enterotoxin and retain pathogenicity (Hasan *et al* ., 1994 ; Garbren *et al* ., 2021)

2.1.2. *Enterobacteriaceae* family

The *Enterobacteriaceae* family includes Gram-negative, motile bacilli (ciliature peritriches) or immobile, asporulate, facultative aero-anaerobes, oxidase negative, catalase positive growing on ordinary media, fermenting the glucose with or without gas production and having a nitrate reductase (exception for certain strains of *Erwinia*) . The genera of this family are *Escherichia , Salmonella* , *Shigella* , *Yersinia* (Berche *et al*., 1988; Leminor et Veron , 1989 ; Hamsatou , 2005).

2.1.2.1. *E. Coli*

E.coli is a gram-negative bacillus known to be a part of normal intestinal flora but can also be the cause of intestinal and extra intestinal illness in humans. There are hundreds of identified *E. coli* strains, resulting in a spectrum of disease from mild, self-limited gastroenteritis to renal failure and septic shock. Its virulence lends to *E. coli's* ability to evade host defenses and develop resistance to common antibiotics. This review will divide *E. coli* infections into those causing intestinal illness and those causing extra intestinal illness. Intestinal illnesses will be described by the causative *E. coli* subtypes, including *enterotoxigenic Escherichia coli* (ETEC), *enter hemorrhagic Escherichia coli* (EHEC) which is also known as *Shiga* toxin-producing *Escherichia coli* (STEC) and will be referred to as EHEC/STEC, enter invasive *Escherichia coli* (EIEC), enter pathogenic *Escherichia coli* (EPEC), and enter aggregative *Escherichia coli* (EAEC). The species most frequently associated with fecal coliforms is *E. coli*, however, represents 80 to 90% of the thermo-tolerant coliforms detected. According to the World Health Organization (WHO) (2004), only states the presence of *E. coli* the indisputable provides proof of pollution. (Dembele, 2005 ; Malberg Tetzschner *et al*., 2020)

2.1.2.2. *Salmonella*

Salmonella are a group of Gram-negative, nonspore forming prokaryotes. Humans are exposed to the pathogen orally, typically through contaminated food or water. Infected individuals may develop an asymptomatic infection, gastroenteritis, or typhoid fever, which may vary in severity, only the latter of which requires antibiotic therapy. *Salmonella* have a number of virulence factors that contribute to disease. (Hamsatou , 2005 ; Adesiyun *et al*.,2020)

Salmonella is a natural inhabitant in the gastrointestinal tract of many animals, including birds, reptiles, livestock, and humans (1–7). *Salmonellosis* caused by nontyphoidal *Salmonella* ranks among the highest in all gastroenteritis cases linked to food consumption, affecting the health of approximately one million people annually in the United States alone (8, 9), resulting in medical costs of $3.7 billion. It is estimated that *Salmonella* species causes 93.8 million cases of gastroenteritis worldwide annually with 155,000 deaths (Liu *et al* ., 2018).

2.1.2.3. *Shigella*

Shigellosis is characterised as an enteric bacterial infection caused by *Shigella spp*. bacteria and is considered a serious health problem worldwide. According to a report of the WHO , *Shigella spp*. is responsible for approximately 165 million cases of shigellosis disease and 1 million deaths annually. *Shigella spp*. is one of the main causes of acute diarrhoea, especially in young children. *Shigella* is a genus of rod-shaped, Gram-negative, facultative anaerobic, non-spore-forming, non-motile bacteria. The genus consists of four known species, including *Shigella dysenteriae* , *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*, which have also been classified serologically as subgroups A–D, respectively. *Shigella sonnei* and *S. flexneri* have been identified as the predominant species and cause enteric infections in several developed and developing countries. (Shahin *et al*.,2019)

2.1.2.4. *Yersinia*

Yersinia enterocolitica is a foodborne pathogen and the causative agent of *yersiniosis*, the fourth most reported zoonotic disease in the European Union , and the fifth most common in the Unites States. The genus *Yersinia* is composed of 19 species, among which two are enter pathogenic to humans (*Y. enterocolitica*, and *Y. pseudotuberculosis*). Enter pathogenic

Yersiniaare mainly found in temperate or cold regions, such as Central and Northern Europe, New-Zealand and North America. Strains of *Yersinia* are ubiquitous and occur in soil, surface water, food and in the digestive tract of various animal species. Y. *enterocolitica* is the main species in the genus associated with *yersiniosis* which can be defined as a mild-moderate self-limiting gastroenteritis. *Y. enterocolitica* is subdivided into 6 biotypes (1A/1B, 2, 3, 4, 5) based on biochemical tests and more than 70 serotypes.*Y. enterocolitica* infection is usually characterized by diarrhea, fever and abdominal pain (Guillier *et al* ., 2020 ; Martins *et al* ., 2021)

2.1.3. Family *Legionellaceae*

2.1.3.1. *L. pneumophila*

The family *Legionellaceae* has more than 50 species and more than 70 serogroups . the L. *Pneumophila* serogroup is the most common. This bacterium grows best on buffered charcoal yeast extract agar, which is a specialized media. It inhibits the growth of other bacteria. The major sources of *Legionella* infection are hot springs and public baths. Among *Legionella* species, *L. pneumophila* serogroup 1 accounts for most human infections. *Legionellosis* outbreaks caused by a combination of *L. pneumophila* serogroup 1 or other serogroups have rarely been reported. . (Füchslin *et al .,* 2010 ; Kourki *et al*., 2017; Mondino *et al*., 2020)

The majority of *Legionnaire's* disease (LD) cases are caused by *L. pneumophila*, a genetically heterogeneous species Persons with underlying health conditions, such as chronic lung disease, or those with compromised immunity are at increased risk for contracting LD (also referred to as *legionellosis*). Signs and symptoms typically include fever, cough, and chest pain; LD is fatal in ≈5%–10% of cases. Transmission of *Legionella pneumophila* is believed to occur mainly through exposure to contaminated aerosols and not from other infected persons; to date, only 1 case of human-to-human transmission has been documented. (Lapierre *et al*., 2017 ; Kozak-Muiznieks *et al*., 2018)

L.pneumophila is the causative agent of *Legionnaire's* diseases. This pathogenic bacterium is ubiquitous in natural aquatic environments such as ponds, lakes, rivers, and estuaries. *L. pneumophila* can be also found in man-made water reservoirs, such as cooling towers, spas , and water distribution systems . Inhalation of water aerosols is the primary

cause of transmission to humans, and human-to-human transmission is rare. (Borthong *et al* ., 2018 ; Ezenarro *et al* ., 2020)

Legionellosis is associated with a mild febrile illness, Pontiac fever, or LD, a cause of severe, atypical, community-acquired pneumonia (CAP). (Gruas *et al* ., 2013)

2.1.4. *Pseudomonadacae* family

The *Pseudomonadacae* family contains Gram-negative bacilli, motile by a polar ciliature (rarely immobile), strict chemo-organothrophs and aerobes. 5 genera are currently included in this family: *Pseudomonas* (type genus), *Xanthomonas, Frateuia, Zoogloea* and *comamonas*. The genus *Xanthomonas* contains isolated phytopathogenic species only in combination with plants. The genera *Frateuria* and *Zoogloea* do not consist of that of a single species, respectively *Frateuria aurantia* and *Zoogloea ramigera* (isolated from wastewater and sludge). Strains belonging to these two genera appear to be saprophytic only (Novotna *et al*.,2019)

2.1.5. *Micrococaceae* family

The Micrococaceae family comprises 3 genera of Gram negative *cocci*, more or less grouped into clusters or tetrads and which differ by their G + C%: *Staphylococcus* (30- 39%), *Micrococcus* (65- 75%) and *Planococcus* (48- 52%). This last kind is not found only in marine bacteriology. The species belonging to these three genera are facultative aerobes, Catalase (+), mobile or immobile, sporulated and showing all tendencies to weightlifting (all grow in the presence of 5% NaCl and many in the presence of 10-15%). The genera of this family are *Micrococcus* and *Staphylococcus* (Shapiro *et al* .,1999 ; Füchslin *et al* ., 2010)

Experimental Part

Chapter 3
Material and Methods

We have analyzed different studies which demonstrated the recents methods used for the detection of pathogenic bacteria in water samples such as : *E.coli , L.pneumophila and V.cholerae* .

3.1. Detection of *E.coli* by Dip Test

3.1.1.Definition of Dip Test

It is a new litmus paper test used for detecting *E. coli* in water samples by performing enzymatic reactions directly on the porous paper substrate. The paper strip consists of a long narrow piece of cellulose blotting paper coated with chemoattractant (at bottom edge), wax hydrophobic barrier (at the top edge), and custom formulated chemical reagents (at reaction zone immediately below the wax hydrophobic barrier). When the paper strip is dipped in water, *E. coli* in the water sample is attracted toward the paper strip due to a chemotaxic mechanism followed by the ascent along the paper strip toward the reaction zone due to a capillary wicking mechanism, and finally the capillary motion is arrested at the top edge of the paper strip by the hydrophobic barrier. The *E. coli* concentrated at the reaction zone of the paper strip will react with custom formulated chemical reagents to produce a pinkish-red color. Such a color change on the paper strip when dipped into water samples indicates the presence of *E. coli* contamination in potable water. (Gunda *et al* ., 2017)

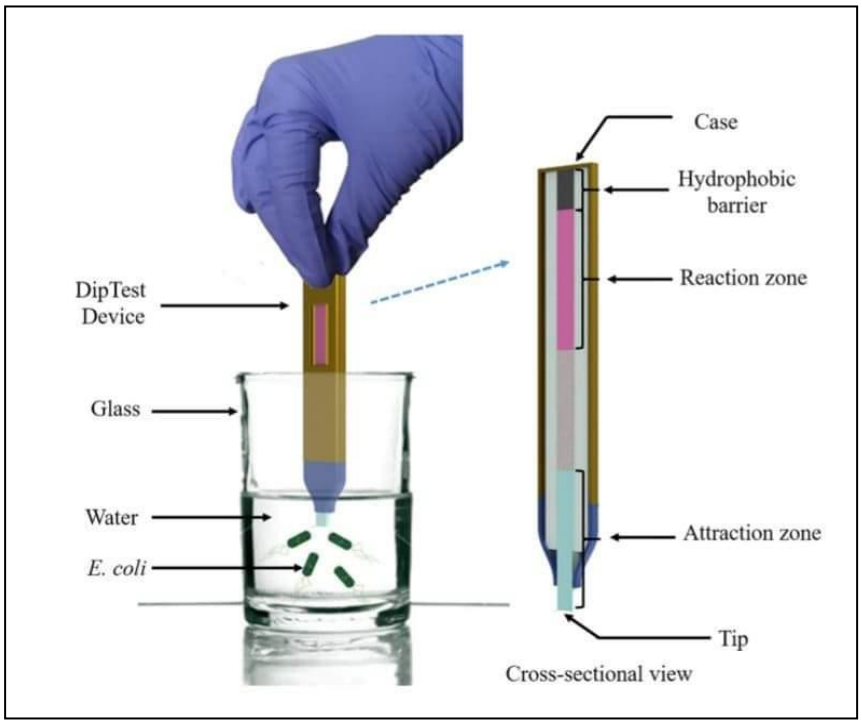

Figure 1. Representation of the use of Dip Test device to test water sample for the presence of *E.coli* bacteria (Gunda *et al* ., 2017)

3.1.2. Material

Whatman gel blotting paper, enzymatic substrate Red-Gal (6-chloro-3-indolyl-β-D-galactoside) and N, N-Dimethylformamide (DMF) were procured from Sigma Aldrich, Canada. Lauryl Tryptose Broth (LTB), Bacteria protein extraction reagent, Veal Infusion Broth , Bacto Yeast Extract , Brain Heart Infusion Broth , and Nutrient Broth were purchased from Fisher Scientific, Canada. (Gunda *et al* ., 2017)

Bacteria strains such as *E.coli* Castellani and Chalmers (American Type Culture Collection (ATCC) 11229), *Enterococcus faecalis* (*E.faecalis*) (ATCC 19433), *Salmonella enterica* (*S.enterica*) (ATCC 14028) and *Bacillus subtilis* (*B.substilis*) (ATCC 33712, MI112 strain) were obtained from Cedarlane, Burlington, ON, Canada. *E.coli* K-12 strains were purchased from New England Biolabs, Ipswich, Massachusetts, USA. (Gunda *et al* ., 2017)

Sodium fluoride, ferric chloride (hexahydrate) and sodium chloride were procured from Fisher Scientific, Canada. Sodium nitrate, iron Chloride hexahydrate, ammonia persulfate, sodium iodide, sodium sulfate, potassium hydroxide, sodium bromide, sodium phosphate, and calcium propionate were purchased from Sigma Aldrich, Canada. Standard

fluoride solution (1ppm), fluoride solution (10ppm), cadmium and lead were obtained from Hanna instruments, Woonsocket, RI, USA. (Gunda *et al* ., 2017)

3.1.3. Methods

3.1.3.1. Preparation of custom formulated chemical composition

Gunda *et al.* (2017) formulated a new chemical composition by dissolving 100 mg of solid media (1:1 mixture of LTB and Red-Gal) in 4 mL of liquid media.The enzymatic substrate Red-Gal is used to detect *E. coli* that secrete β-galactosidase enzymes. A chromogenic compound Red-Gal (6-Chloro-3-indolyl-β-D-galactoside) contains two components: 6-Chloro-3-indolyl and β-D-galactoside. The β-galactosidase enzyme produced by *E. coli* hydrolyses this complex Red-Gal molecule resulting in the release of pinkish red color producing dimerized 6-Chloro-3-indolyl compound. The inclusion of Bactérial Protein Extraction (B-PER) in custom formulated chemical reagents is to accelerate the extraction of β-galactosidase enzymes by lysing the *E. coli* bacteria cells without denaturing the bacterial enzymes. (Gunda *et al* ., 2017)

3.1.3.2. Preparation of DipTest device

Initially, the blotting paper is diced into 70 mm × 5 mm size strips. The length of paper strip chosen i.e. 70 mm is enough for the capillary imbibition to occur. Blotting paper is made of pure cellulose produced entirely from the high quality cotton linters with no additives. Blotting paper has a weight of 320 g/m^2, wet strength of 300 mm water column and water absorbency of 740 g/m^2. The blotting paper ensures the proper wicking and uniform capillary action. One edge of the paper strip is coated with wax to form a hydrophobic barrier. The wax barrier prevents the further spreading of the chemicals and bacteria in the reaction zone through capillary action. The reaction zone is formed below the hydrophobic barrier by depositing the 100 μL of above mentioned custom formulated chemical composition (Red-Gal, B-PER and LTB) using pipette and followed by drying under normal laboratory condition (temperature around 23°C) for one hour. After coating custom formulated chemical composition at the reaction zone, the opposite edge of the paper strip is coated with D-glucose (dextrose) by dispensing 100 μL of 0.1 M D-glucose and then allowed to be dried at room temperature (23°C) for one hour. This edge is also known as attraction zone since D-glucose acts as a chemotaxis agent to attract the bacteria towards the paper strip. The resulting paper

strips were completely dried for one hour under a fume hood before dipping them into *E. coli* contaminated water. (Gunda *et al* ., 2017)

3.1.3.3. Testing water samples with DipTest device

To perform the test, the edge with attraction zone of DipTest device needs to be dipped into the *E. coli* contaminated water. The D-glucose in the attraction zone gets dispersed and forms a concentration gradient in the water. This gradient creates the chemotactic movement of *E. coli* bacteria from the surrounding water and it eventually increases the migration of bacteria towards the paper strip . The water along with bacteria (attracted at the edge of the paper strip) percolates into the porous matrix of paper strip due to capillary action. Once the water front reaches the hydrophobic barrier on a paper strip (DipTest), the DipTest is removed from the water and kept aside on a flat surface. The bacteria trapped in the reaction zone will react with chemicals and produce the pinkish red color. The appearance of pinkish red color indicates the presence of *E. coli* bacteria. It is to be noted that all the tests with DipTest are conducted at room temperature. (Dasgupta *et al* ., 2016 ; Gunda *et al* ., 2017) .

3.2. Detection of *L. pneumophila* by droplet digital PCR (ddPCR)

3.2.1. Definition of droplet digital PCR

Digital PCR is a novel molecular method enabling absolute quantification of deoxyribonucleic acid (DNA) targets without the need to construct a calibration curve as used commonly in quantitative PCR (qPCR). The principle of digital PCR was first introduced in the 1990s and the recent development of ddPCR has been used widely in medical researches and clinical applications. The ddPCR approach partitions a bulk fluorescent PCR reaction containing DNA templates, primers and a fluorescently labeled hydrolysis probe or a nucleic acid intercalating dye (EvaGreen) into thousands of nanoliter-sized water-in-oil microdroplets. Target DNA and background DNA are distributed randomly among these droplets. Every microdroplet is a micro PCR reactor, with each containing zero or at least one copy of the target DNA. After emulsion PCR to the end-point, droplets are analyzed individually by a mechanism similar to flow cytometry. Fluorescent and non-fluorescent droplets are defined as positive (presence of target sequence) or negative (absence of target sequence), respectively. (Sykes *et al.,* 1992 ; Vogelstein et Kinzler , 1999 ; Hindson *et al*., 2011)

As an emerging versatile molecular biotechnology, ddPCR is a robust and powerful method for the detection and quantification of nucleic acids with unparalleled accuracy and precision without the need for an external calibration curve or reference. ddPCR is rapidly replacing qPCR as an efficient method for independent DNA quantification. In recent years, there have been increasing numbers of applications**)** of the ddPCR assay used in medical, environmental and food safety control applications (Huggett *et al* ., 2013 ; Miotke *et al*., 2014 ; Cao *et al* .,2015 ; Bucher et Köppel , 2016)

An innovative technology, ddPCR used to track mutations of interest over time. Features of this approach are ultrahigh sensitivity (up to the 0.001% mutated allele frequency) and higher precision than conventional qPCR assays, which can achieve similar results only through the use of multiple replicates. ddPCR to be a highly sensitive platform, enabling absolute quantitation of mutant BRAF (v-raf murine sarcoma viral oncogene homolog B1) down to 0.001% allelic fraction (Pinheiro *et al* ., 2012 ; Brambati *et al .,* 2016 ; Mcevoy *et al*., 2017)

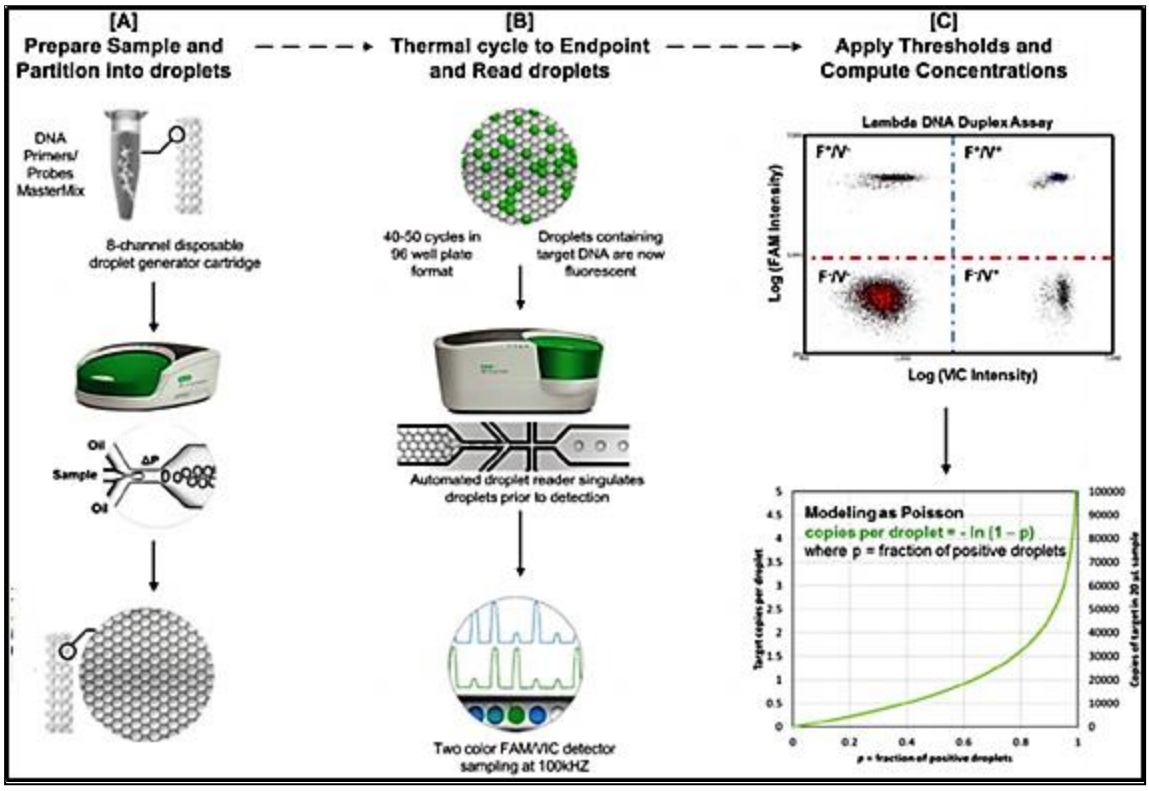

Figure 2.Steps of ddPCR technique (site web 01)

3.2.2. Material

- Glycine Vancomycin Polymyxin Cycloheximide (GVPC) medium
- liquid broth
- distilled water
- antimicrobial agents
- *L. pneumophila* culture
- flasks
- Sybricons (SYBR-Green), Reverse transcription-quantitavie polymerase chain reaction (RT-qPCR)
- Ethylene Vinyle Acetate (EVA) Green
- ddPCR.
- the QX200 droplet generator
- A 96-well plate
- 2X QX200™ ddPCR™ Eva-Green Supermix
- RNase and DNase free-water
- cDNA
- C1000 Thermal Cycler
- The QuantaSoft.
- GraphPad Prism V.6.
- The Pure Link Genomic DNA Mini (Falzone *et al* ., 2020)

3.2.3. Methods

3.2.3.1. Bacterial strain and culture conditions

L. pneumophila serotype 1 was obtained from an environmental contaminated water site. Serotype 1 was identified by using lactic test.

L. pneumophila was grown on liquid broth and then on GVPC medium, corresponding to buffered charcoal yeast extract medium plus antimicrobial agents, at 37°C in a 5% CO_2-enriched atmosphere for 5 days.

- After growth, single colonies were resuspended in sterile water until a turbidity of 0.5 McFarland (1.5×10^8 Colony forming unit (CFU) /ml) was obtained. (Falzone *et al* ., 2020)

3.2.3.2. Preparation of dilution and DNA extraction for *L. pneumophila*

A dilution of 10^7 CFU/ml was obtained starting from a concentration of 1.5×10^8 CFU/ml.

10-fold serial dilutions in sterile water were performed until a concentration of 10 CFU/ml was obtained. The 10-fold serial dilutions were used to assess the sensitivity of both methods.

In parallel, *L. pneumophila* was seeded into two flasks with sterile water with a final concentration 10^2 CFU/ml.

One of the two flasks was subjected to thermal shock at 80°C for 30 min for 3 consecutive days in order to kill *L. pneumophila*.

Different samples were obtained at different time points from the two flasks in order to assess the efficacy of thermal shock and the sensitivity of both ddPCR and Reverse transcription - polymerase chain reaction quantitative RT-qPCR **(Fig 03).**

Then, 1 ml of each dilution or sample obtained from the two flasks was extracted using the Pure Link Genomic DNA Mini kit extraction kit.

- The extracted DNA was quantified by using spectrophotometric assay . (Falzone *et al* ., 2020)

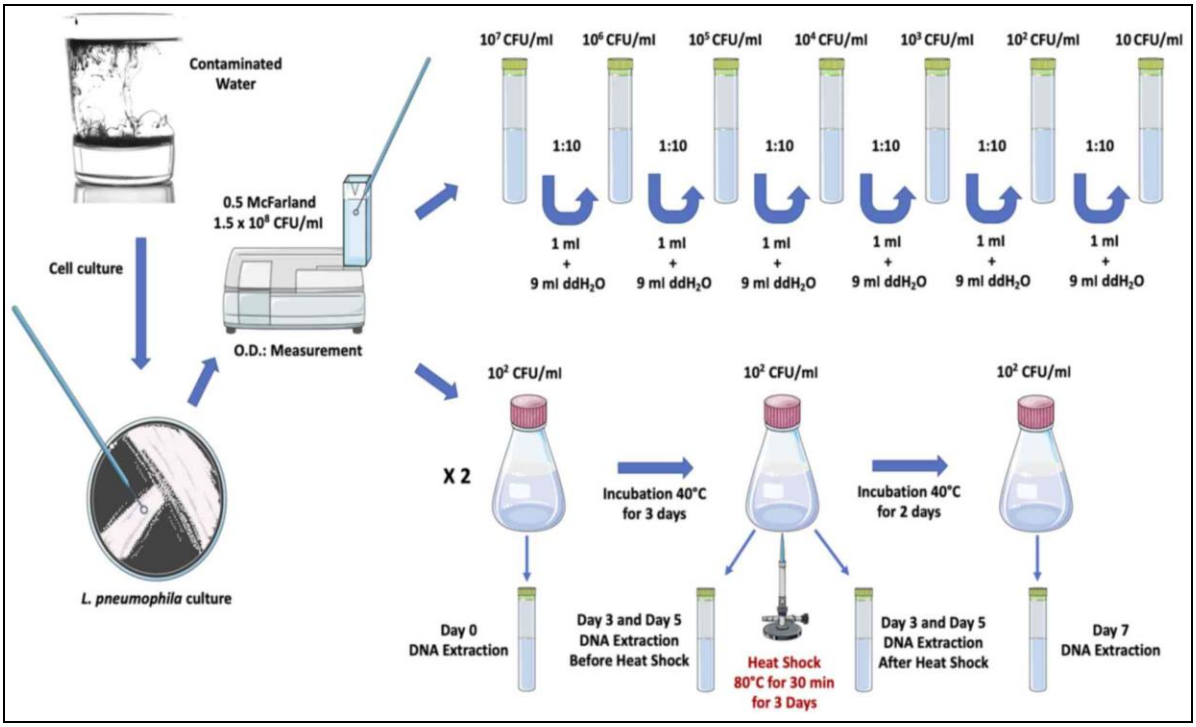

Figure 3.Préparation of different samples in order to assess the éfficacity of thermal shock and sensivity of both ddPCR and RT-Qpcr. (Falzone *et al* ., 2020)

3.2.3.3. *L. pneumophila* RT-qPCR and ddPCR amplification

Extracted DNA (4.7 μl) was amplified by using both SYBR-Green RT-qPCR and Eva-Green ddPCR.

For RT-qPCR: the Luminaris Color HiGreen qPCR Master Mix, high carboxy-X-rhodamine (ROX) was used according to the manufacturer's protocol.

L. pneumophila were amplified with a 7300 Real-Time PCR System using the following primer pairs and thermal conditions: forward: AGGGTTGATAGGTTAAGAGC; reverse: CCAACAGCTAGTTGACATCG; RT-qPCR thermal profile: UDG pre-treatment at 50°C for 2 min, followed by an initial denaturation step at 95°C for 10 min and a 3-step PCR program at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec, for 40 cycles. The selected primers are specific for all *L. pneumophila* serogroups. (Falzone *et al* ., 2020)

For Eva-Green ddPCR, the reaction mix was prepared by using 11 μl of 2X QX200™ ddPCR™ Eva-Green Supermix, 0.15 μl of 20 μM forward and reverse primers (same primers used for RT-qPCR), 6 μl of RNase and DNase free-water and 4.7 μl of cDNA in order to obtain a final volume of 22 μl. (Falzone *et al* ., 2020)

20 μl of the reaction mix were used to generate droplets with the QX200 droplet generator.

After generation:

The droplets were transferred into a 96-well plate, sealed and amplified in a C1000 Thermal Cycler under the following thermal conditions: polymerase activation at 95°C for 10 min, 40 cycles of amplification at 94°C for 30 sec (denaturation) and 60°C for 1 min (annealing/elongation), droplets stabilization at 98°C for 10 min followed by an infinite hold at 4°C. (Falzone *et al* ., 2020)

- A ramp rate of 2°C/sec was used among the steps of the amplification (Falzone *et al* ., 2020)

3.3. Detection of *V. cholerae* by hybridoma

3.3.1. Definition of hybridoma technology

Hybridoma technology has been almost 40 years since monoclonal antibodies were first generated by Georges Kohler and César Milstein in 1975 by fusing mouse myeloma cells and mouse splenocytes it has long been a remarkable and indispensable platform for generating high-quality monoclonal antibodies (mAbs). Hybridoma-derived mAbs have not only served as powerful tool reagents but also have emerged as the most rapidly expanding class of therapeutic biologics**.** The mAbs generated from this technology have served as

reagents for the identification and characterization of cell surface antigens, for classification and isolation of hematopoietic cell subsets, and for the development of biomarkers to distinguish aberrant or cancerous cells from normal cells (Zhang, 2012 ; Holzlohner et Hanack, 2017)

Hybridoma technology relies on B cells that are matured in secondary lymphatic organs in response to an antigen. These B cells undergo natural antibody maturation process where the variable region of antibodies diversified by accumulating somatic hypermutations which further results in the selection of high-affinity tight binders . Resulting antibodies possess the natural pairing of variable heavy and light chain genes with naturally class-switched matured constant region gene through class switch recombination (CSR). Such freedom of natural CSR is not possible in other mAb isolation method that makes hybridoma a unique way to produce naturally matured in vivo antibodies in the laboratory. Generally, there are two types of hybridomas: one is homo-hybridomas and second is hetero-hybridomas. (Parray *et al .,* 2020).

In homo-hybridomas both, the immunoglobuline G (IgG) secreting by B cells and fusion partners are from the same species. In hetero-hybridomas the antibody-secreting B cells and fusion partners are from two different species. Homo hybridomas are genetically more stable and secrete stable IgG as compared to hetero-hybridomas as it gradually lost the chromosomal recombinants during the clonal selection step due to their genetic instability. (Parray *et al .,* 2020).

3.3.2. Three major steps of Hybridoma technology

The first step of MAb production is **stimulation of the animal immune system** ; Following the optimization, a host animal is immunized with the Ag along with adjuvant for several weeks. This injected antigen mixture is slowly released and travels up to the mouse perithymic lymph node, leading to activation of antibody secreting plasma cells. (Kim *et al*., 2014; Parray *et al*., 2020)

The second step in MAb production is **immortalization**. This is achieved in one of two ways: (a) constraints of lymphoid cells and (b) cell transformation. Today, the most common immortalization method utilizes genetic information obtained from transformed or immortalized myeloma cell lines. (Kim *et al*., 2014; Parray *et al*., 2020)

The third and final step of MAb production is **isolation** of single antibody-producing cells. This is done by elimination of the unfused parent cell types. After determining which culture supernatants are of interest, these cells are harvested, diluted, and then replated.MAbs produced by a single clone of cells, having a homogeneous binding specificity for an antigenic determinant, can be further characterized and used as a specific reagent for diagnostics or therapeutics **(Fig 4)** (Kim *et al*., 2014; Parray *et al*., 2020) .

The spleen cells are fused with the immortalised myeloma cells in the presence of fusogenic agents like viruses, chemicals and electric pulses. The fused cells are then selected on hypoxanthine-aminopterin-thymidine medium. The myeloma cells are sensitive to hypoxanthine aminopterin thymidine (HAT) medium as they lack hypoxanthine-guanine phosphoribosyl transferase gene required for nucleotide synthesis by the de novo or salvage pathways while the unfused B cells die as of short life span. In this process, only the hybrid survives, as they harbour the functional hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene from the B cells. However, hybrid cells retain the dual properties, antibody secreting property of B cells and continuously growing property from myeloma cells. Fused or hybrid cells are then screened by «limited dilution cloning» method or with semi solid selective medium to select only those hybridoma that produce antibodies of appropriate specificity. (Parray *et al* ., 2020).

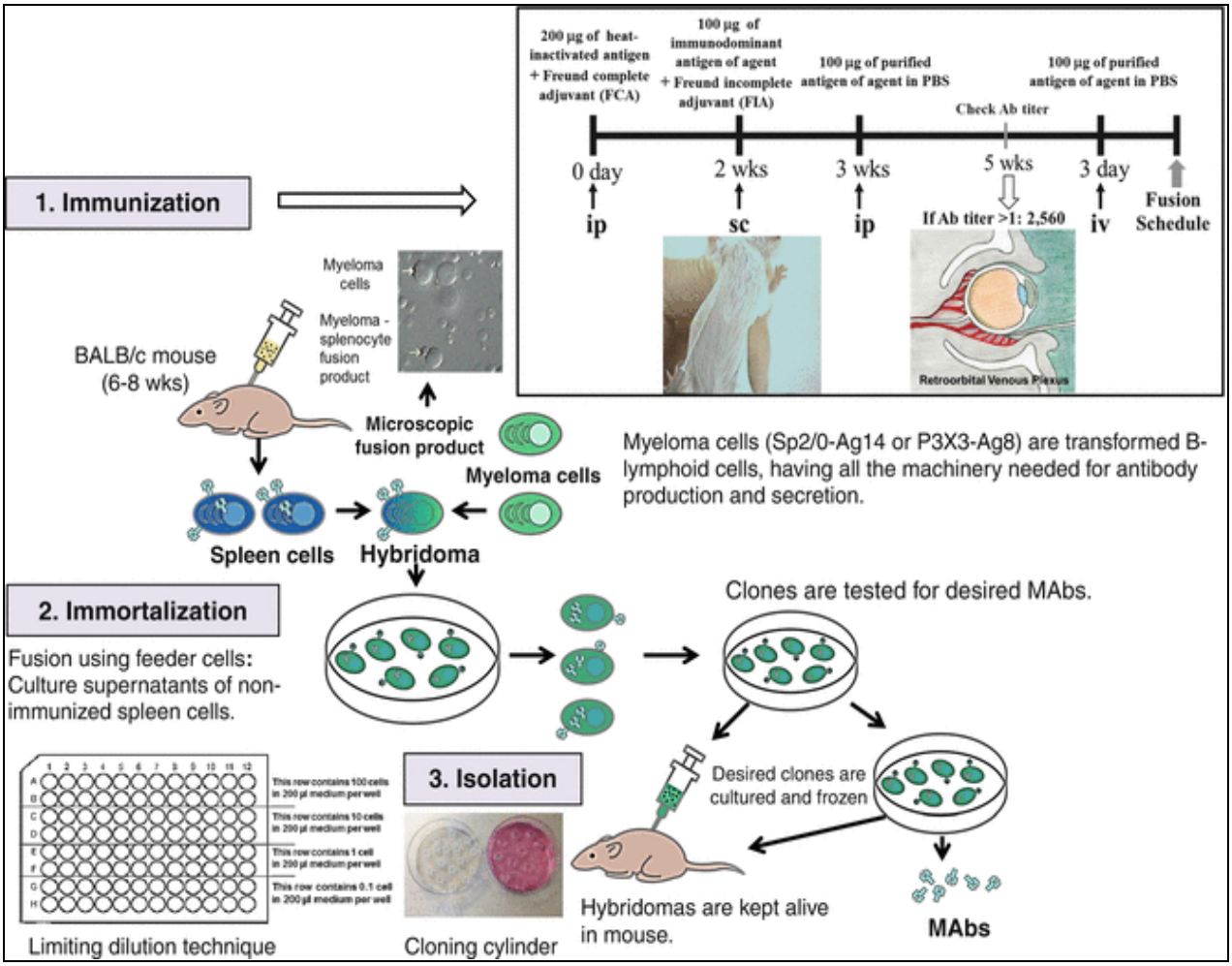

Figure 4. Three major steps of hybridoma technique (Kim *et al* ., 2014)

3.3.3. Fluorescence hybridoma-based assay for *V. cholerae* detection

Hybridoma cells were exposed to an efficient fluorescence calcium indicator, fura 2-AM. Binding of *V. cholerae* antigen to specific antibody on the surface of the hybridoma cells initiates a signaling pathway that increases cytosolic calcium, causing the fluorescence probe to emit light. Taking a broader view, after antigen-antibody binding, receptor tyrosine kinases activate the phospholipase C (PLC) enzyme, which hydrolyzes the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 is a crucial second messenger involved in cell signaling pathway, which releases Ca^{2+} from the endoplasmic reticulum and activates calcium channels to release calcium into cytoplasm **(Fig.05) .** (Zamani *et al* ., 2016)

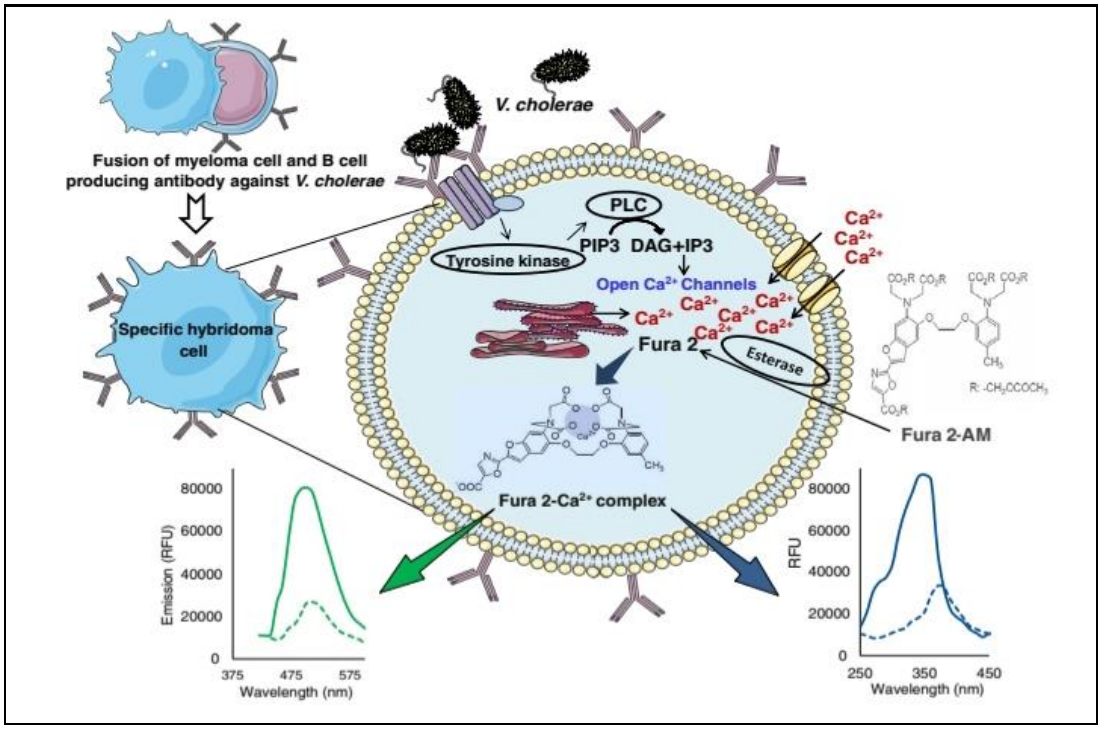

Figure 5.Shematics representation of flurescence hybridoma based assays for *v.cholerae* detection (Zamani *et al* ., 2016)

3.3.4. Material

An efficient fluorescence calcium indicator fura 2- acetoxy methyl (Fura 2-AM) ; Roswell Park Memorial Institute (RPMI) 1640 medium. ; fetal bovine serum (FBS) ; Phosphate-buffered saline (PBS) ; Cell culture reagents ; Penicillin-streptomycin. ; Dimethyl sulfoxide (DMSO), microbiological medium, and other chemicals . also T75, T25 flasks, and 96 wall plates (Zamani *et al* ., 2016)

3.3.5. Methods

3.3.5.1. Bacterial strains and growth conditions

The *V. cholerae* were selected for the project ATCC 39315, 14034, PTCC 1611 (Inaba O1), and *V. cholerae* ATCC 14035 (Ogawa O1) (Zamani *et al* ., 2016)

The specificity of the assays is determined by using a series of bacteria, including *V. harveyi* (PTCC 1755) , *V. fischeri* (PTCC 1693), *V. vulnificus* (ATCC 27562), *V.parahaemolyticus* (ATCC 17802), *V. mimicus* (ATCC33655), *V. cholerae* O139 (ATCC 51395, 51394), *V. Cholera* non-O1 (ATCC 25872, 25874, 35971, 14374), *V. cholerae* O7 (ATCC 14733), *V. cholerae* O3 (14731), *V. cholerae* O2 (14730), *Escherichia coli* (ATCC

25922), *Entrobacter cloacae* (PTCC 1003), *Citrobacter freundii* (NCIMB 11490), *Shigella sonnei* (ATCC 9290), *Salmonella typhi* (PTCC 1609), and *Pseudomonas aeruginosa* (ATCC 27853) as negative controls. (Zamani *et al* ., 2016)

The bacterial strains are maintained at 37 °C for 12 h in lysogeny broth (LB) medium consisting of 10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract. (Zamani *et al* ., 2016)

3.3.5.2. Cell culture

Hybridoma cells with immunoglobuline M (IgM) antibody against lipopolysaccharide (LPS) of *V. cholerae* O1) using fusion of immunized spleen cell with mouse myeloma SP2/0 cells. (Zamani *et al* ., 2016)

Mouse myeloma SP2/0 and MC2B8 hybridoma (a specific hybridoma cell line that produces monoclonal antibody against plasminogen) were used as negative controls for the following assay: The cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10 % fetal bovine serum(FBS) and 50 µg/mL penicillin-streptomycin at 37 °C and 5 % CO2. (Zamani *et al* ., 2016)

Cells were grown to approximately 80 % confluence for the experiments.

They were transferred to FBS containing 10% DMSO and frozen in liquid nitrogen for long-term storage. (Zamani *et al* ., 2016)

3.3.5.3. Intracellular calcium measurement using fura 2-AM

Cells were washed with PBS and then 2 µM of fura 2-AM was added to them and they were left in the dark for 30 min at 37 °C. (Zamani *et al* ., 2016)

After washing, the cells were incubated for an additional 15 min at 37 °C to allow complete ester hydrolysis. (Zamani *et al* ., 2016)

Following extensive washing, cells were exposed to different concentrations of bacteria, and the fluorescence emission was immediately recorded using Synergy Multimode Reader . For each experiment, cells were exposed to 340 nm wavelength to record emission at 505 nm. Following the light pulse, more data were obtained at 340 and 380 nm and the ratios of 340/380 wavelengths were determined within 10 min of loading. For determining the appropriate cell number, the cells were grown to 80 % confluence in tissue culture flasks; then up to 106 cells were used and the data were recorded at 10 min after adding 200 CFU/mL of bacteria. (Zamani *et al* ., 2016)

3.3.5.4. Evaluation of method specificity and detection limit

Two separate experiments were designed as follows:

- In the first experiment:

3 × 105 specific cells were exposed to fura 2-AM as mentioned above. Next, the hybridoma cells were treated with *V. cholerae* O1 (200 CFU/mL) and 2000 CFU/Ml of different bacteria, including V. *mimicus, V. vulnificus, V. parahaemolyticus, V. fischeri, V. harveyi, V. cholerae* O139, O2, O3, O7, and non-O1 *Vibrio cholerae*, environmental water samples (samples with O1 and non-O1 *V. cholerae*), *Salmonella typhi, Shigella sonnei, E. coli, Entrobacter cloacae, Citrobacter freundii,* and *Pseudomonas aeruginosa*. Next, fura 2-AM emissions were monitored on 505 nm. (Zamani *et al* ., 2016)

- In the second experiment:

3 × 105 cells of specific hybridoma and negative controls were treated with 500 CFU/ml of *V. cholerae* O1 and fura 2-AM fluorescence emission were recorded. The dose response curve was established by adding variable concentrations of *V. cholerae* O1 from 0 to 1000 CFU/ mL to hybridoma cell suspension.
The Electric Conductivity EC50 values and 95 % confidence limit were calculated using POLO-PC program. (Zamani *et al* ., 2016)

3.3.5.5. Spiked environmental sample analysis

Environmental samples were prepared using fresh water, waste water, and sea water spiked with serial dilutions of *V. Cholera* O1. The CFU of bacteria added to spike the water samples was estimated by plating serial dilutions of the initial inoculum. And, for preparing spiked stool, a fecal specimen from a healthy individual was diluted 5-fold with physiologic sodium chloride solution and then different CFU of *V. cholerae* O1 was added. All the assays were conducted and fluorescence emissions were recorded at 505 nm. (Zamani *et al* ., 2016)

Chapter 4
Results and Discussions

4.1. Detection of *E.coli* by Dip Test

4.1.1. Results

Figure (6) below illustrates the color change at the reaction zone of DipTest device because of the presence of a known concentration of *E. coli* in contaminated water. It is observed that there is a pinkish red color at the reaction zone of the DipTest device, which represents the presence of *E. coli*. A controlled study is conducted where DipTest device was tested in DI water at room temperature with no *E. coli* and it is found that there is no color change in the reaction zone. The inset of **Fig (6a)** shows the scanning electron microscope (SEM) image of the porous paper matrix. It is observed that the paper is randomly distributed network of paper fibres with an estimated porosity of 65 to 73%. (Gunda *et al* ., 2017)

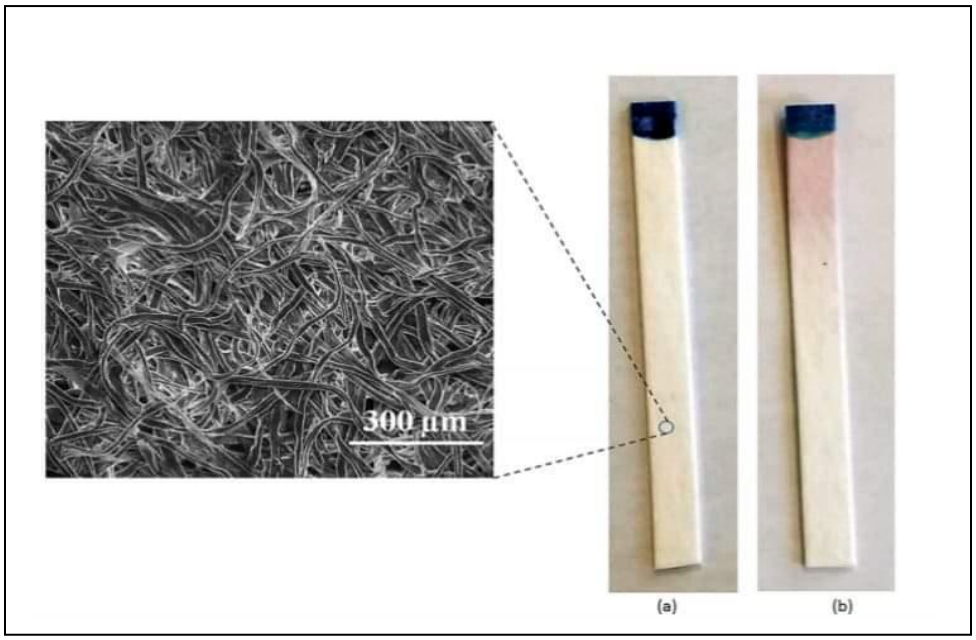

Figure 6.Comparison of Dip Test device between (a) tested with DI water at room température (b) tested with *E.coli* contaminated water at room temperature (Gunda *et al* ., 2017)

Figure (7) shows the appearance of pinkish red color at the reaction zone of the DipTest device for various known concentrations of *E. coli* contaminated water samples after 2 hrs at room temperature. It is to be noted that the color intensity varies based on the concentration of bacteria in water samples and how much time the DipTest device is dipped into the water. It is found that the color intensity decreases with the decrease in the concentration of *E. coli*. This figure also shows the sensitivity and limit of detection of DipTest device. The present method

could detect up to 200 CFU/mL and this can be considered as the limit of detection (LOD). (Gunda *et al* ., 2017)

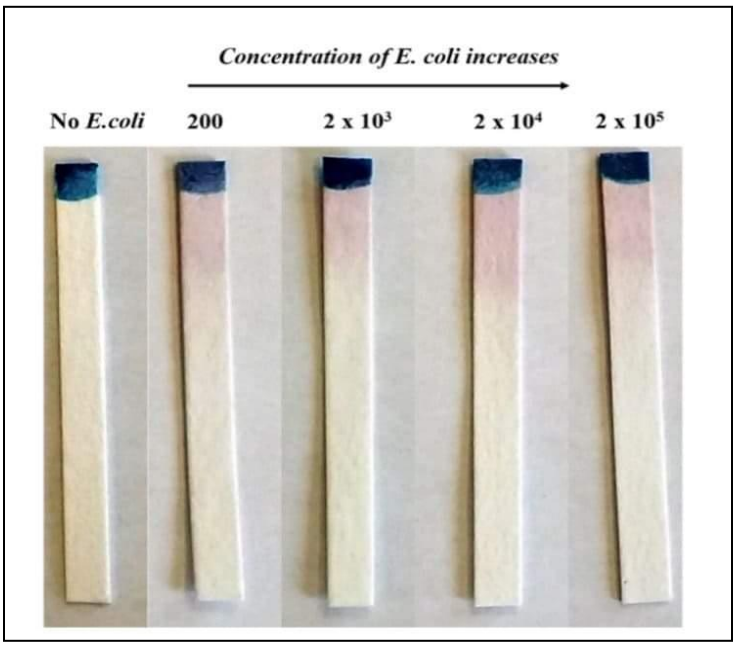

Figure 7.Development of prinkich red color on Dip Test device after 2 hrs based on the concentration of *E.coli* (CFU/ml) . (Gunda *et al* ., 2017)

The DipTest device performance is verified for its specificity by testing the device with several water samples containing different interfering bacteria and chemical contaminants Table (2) shows the DipTest results for 40 different water samples and containing several interfering bacteria. (Gunda *et al* ., 2017)

The results demostrased that the custom formulated chemical composition coated on DipTest device is the main component which ensures the specificity to detect *E. coli* and contains from the favorable growth medium i.e. LTB and enzymatic substrate (Red-Gal). LTB favors the maximum possible growth of *E. coli* as well as the maximum possible production of the *β*-galactosidase enzyme. The produced *β*-galactosidase enzyme hydrolyzes the Red-Gal and thereby produces the pinkish red color. (Gunda *et al* ., 2017)

Table 2.Dip Test results for different water samples used in this study (Gunda *et al* ., 2017)

Category	Sample Number	Contents in water samples	Dip Test (Dip time :90 min ; wait time :180 min)
A	1	*B.Subtilis*	No color
	2	*S.Entérica*	No color
B	3	ATCC11229, *E.coli* K-12 and *B.Subtilis*	Color produced
	4	ATCC11229, *E.coli* K-12 and *S.Entérica*	Color produced
C	5	Sodium Fluoride	No color
	6	Sodium Nitrate	No color
D	7	ATCC11229, *E.coli* K-12 and Sodium Fluoride	Color produced
	8	ATCC11229, *E.coli* K-12 and Sodium Nitrate	Color produced

4.1.2. Discussion

Currently, for a dip time of 2 min, DipTest device is able to detect as low as 200 CFU/mL in 180 ± 20 min and higher concentrations such as 2×10^5 CFU/mL within 75 ± 12 min. However, for a dip time of 90 min, DipTest device is able to detect as low as 200 CFU/mL in 54±8 min and higher concentrations such as 2×10^5 CFU/mL within 28±5 min. The performance of DipTest device is checked and verified under different kind of water samples containing interfering bacteria and chemical contaminants. This DipTest device would eventually act as a field screening test that can be carried in a pocket and one can conduct the testing of water samples whenever required. The DipTest device can also be disposed off easily after completion of test with minimal effort. Further optimizations in terms of the concentration of individual chemical ingredients used here are needed so that one can eventually have a field deployable device to provide “yes/no” litmus test

for *E. coli* concentration as low as 1–10 CFU/100 mL, thereby meeting the United States Enviromental Protection Agency (US EPA) standards. The current DipTest platform can be adapted and integrated with further developments in the detection of other bacteria and pathogens and used not just for water samples but for many other products (milk, wine, juices, etc.) and food industry (frozen meat and cheese). (Gunda *et al* ., 2017)

Chen *et al.* (2015) used T7 bacteriophage- conjugated magnetic beads to detect low levels of *E. coli* in drinking water. The T7 phage can specifically recognize and bind to *E.coli* and the magnetic beads are used to separate and concentrate the bacterial cells from drinking water. After concentration and separation, phages will infect and lyse the host cells resulting in the release of β-galactosidase .The released enzyme can then be readily detected using a colorimetric substrate" chlorophenol red-β-d-galactopyranoside" (CRPG) which changes from yellow to red in the presence of this enzyme .

This technique is able to detect *E. coli* at a concentration of 1×10^4 CFU·mL^{-1} in 2.5 hours and 10 CFU·mL^{-1} of *E. coli* in drinking water can be detected after pre- enrichment for 6 hours. (Chen *et al* ., 2015)

The assay has been designed to allow portability with the use of minimal equipment and This will allow the rapid determination of *E. coli* concentrations in water in settings where proper laboratories are not available . (Chen *et al* ., 2015)

Gunda *et al.* (2016) used a novel hydrogel based porous matrix to encapsulate the optimized chemical compounds and incorporated it within a readily available plunger-tube assembly. This overall system allows efficient; field deployable and rapid testing of water samples by simultaneously pre-concentrating and detecting *E. coli* within one integrated unit.

The plunger-tube assembly method allows the user to detect *E. coli* by visualizing the appearance of pinkish red color within minutes of testing contaminated water and this method does not require any microbiology instruments and trained personnel . The ease of the testing method makes it a potential candidate for field deployment in limited resource communities. (Gunda *et al* ., 2016)

Additionally , It's ablility to detect *E. coli* concentrations of 4×10^6 CFU mL^{-1} to 4×10^5 CFU mL^{-1} within 5 min and 4×10^4 CFU mL^{-1} to 400 CFU mL^{-1} within 60 min using the integrated plunger-tube assembly containing the hydrogel matrix . (Gunda *et al* ., 2016)

Ngamsom *et al.* (2017) presented a simple microfluidic system for rapid screening of E.coli O157:H7 employing the specificity of immunomagnetic separation (IMS) via immiscible filtration assisted by surface tension (IFAST) and the sensitivity of the subsequent adenosine triphosphate (ATP) assay by the bioluminescence luciferin/luciferase reaction. The developed device is capable of detecting *E. coli* O157:H7 from just 6 colony forming units in 1 mL spiked buffer within 20 min .

Wu *et al.* (2018) innovated a membrane filter-based approach to facilitate more rapid enzyme-based detection of *E.coli* in water based on the combination of an initial concentration step and optimized test conditions. For this approach, a water sample (10–100 mL) is filtered through a 0.45-μm pore size filter with a diameter of 4 or 13 mm. After filtration, a newly designed rapid detection broth is added containing the enzymatic inducer Methyl-beta-D-Glucuronide sodium (MetGlu) and the substrate 5-Bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) or Resorufin β-D-glucuronide (REG) . After a few (1–7) hours of incubation at 35 °C, the filter shows pink color (for REG-containing broth) or green color (for X-Gluc containing broth) indicating the presence of *E.coli* and the results can be observed with naked eye.

The new approach with the substrate X-Gluc or REG has several advantages over other substrate specific methods and is amenable to more rapidly detecting and quantifying the presence of *E. coli* in water in resource limited areas . (Wu *et al* ., 2018)

Tok *et al.* (2019) presented an automated device which can test drinking water samples for *E. coli* and using an Enviromental protection agency (EPA)-approved reagent. Equipped with a Raspberry Pi microcontroller and camera which can perform automated periodic measurements of both the absorption and fluorescence of the water under test over 24 hours.

This device weighs 1.66 kg which can automatically detect the presence of both *E. coli* in drinking water within less than 16 hours, down to a level of one colony forming unit per 100 mL. Furthermore, due to its automated analysis, this approach is also more sensitive than a manual count performed by an expert, reducing the time needed to determine whether the water under test is safe to drink or not. (Tok *et al* ., 2019)

This device is rather cost-effective with its parts costing 600 Dollars under low volume manufacturing which can be significantly reduced with economies of scal . (Tok *et al* ., 2019)

Therefore, it is applicable in a variety of settings particularly in areas where access to a central lab or transportation of the sample are not feasible. (Tok *et al* ., 2019)

Wibowo *et al.* (2020) reported on biocompatibility of graphene against *E. coli* through visible growth inhibitory test and SEM image that showed no inhibition growth and undamaged bacteria cell. Graphene wettability over water contact angle performed by hydrophobicity with 108.90° which preferably attached by the cells due to its surface energy condition. Negativity charge of bacteria enables to induce the holes of graphene then formed ohmic contact between the sheets and electrode. Based on its resistance, graphene could detect *E. coli* with limit of detection 4 CFU. Therefore, a compact and portable design of *E. coli* sensor was fabricated with the device average error about 12.23%.

Romão *et al.* (2020) developed a portable culture device based on the point of care (POC) premises , seeing as it is produced using simple analytical components which can detect *E.coli* by colorimetry because chromogenic culture medium added which contains enzymatic components able to react and release a stable chromogen in the presence of specific enzymes from the target bacterial groups .

This method can detect *E.coli* after few hours from incubation and the limit of detection is 1 CFU/mL .It's limited by the use of liquid samples with low volumes but high volumes of samples can overflow the hydrophilic limits of the culture device . (Romão *et al* ., 2020)

Additionally, the platform has a low cost and is developed with materials that are easy and quick to manufacture and to analyze, thus making it a great alternative for the fast and reliable pathogen identification. This is important to improve the quality of life and maintain the health of populations that they live in regions that are difficult to access or that need to travel great distances to have access to laboratory infrastructure, which is very common in developing countries. (Romão *et al* ., 2020)

4.2. Detection of *L. pneumophila* by ddPCR

4.2.1.Results

4.2.1.1.Comparison of specificity of ddPCR and RT-Qpcr

The serial dilutions obtained were used to assess the sensitivity of both ddPCR and RT-qPCR systems by using Eva-Green and SYBR-Green technologies, respectively. (Falzone *et al*., 2020)

The obtained results showed that both methods are sensitive enough to detect the presence of *L. pneumophila* at concentrations established by law (10 CFU/ml, i.e., 10^2 CFU/l if the standard protocol for *L. pneumophila* detection starting from a 1 liter filtered water sample is used). (Falzone *et al*., 2020)

However, the signal related to the sample diluted at 10 CFU/ml was obtained at a very late Cycle threshold (Ct) value when RT-qPCR is used, while ddPCR effectively detects as positive the signals obtained for the same concentration **(Table 3)** (Falzone *et* al., 2020)

Table 3.RT-qPCR Ct values and ddPCR *L.pneumophila* absolute quantification (Falzone *et al*., 2020)

No.	**Rt-qPCR**		**ddPCR**	
	Sample	**Ct**	**Sample**	**Copies/µl**
1	10^7 CFU/ml	11.26	10^7 CFU/ml	2,298
2	10^6 CFU/ml	15.02	10^6 CFU/ml	1,762
3	10^5 CFU/ml	17.75	10^5 CFU/ml	913
4	10^4 CFU/ml	22.62	10^4 CFU/ml	91.60
5	10^3 CFU/ml	25.64	10^3 CFU/ml	9.30
6	10^2 CFU/ml	28.06	10^2 CFU/ml	1.40
7	10 CFU/ml	31.10	10 CFU/ml	0.29
8	NTC	-	NTC	-

NTC : No template control **, Ct :** Cycle threshold, **RT-qPCR** :Reverse transcription-quantitative polymerase chain reaction , **ddPCR :**droplet digital polymerase chain réaction

Noteworthy, the absolute quantification performed by ddPCR showed that the copies/µl obtained for each sample better reflects the 10-fold serial dilutions performed, except for the concentration of 107 CFU/ml that was underestimated due to the high number of positive droplets that saturated the ddPCR system. In addition, linear regression analysis showed that ddPCR has a greater accuracy and robustness compared to RT-qPCR. By excluding the 107 CFU/ml concentration that saturated both ddPCR and RT-qPCR systems, linear regression analysis revealed that ddPCR has a better r2 coefficient compared to RT-qPCR (r2=0.8388 vs. r2=0.5228) **(Fig 8.)** (Falzone *et al.*, 2020)

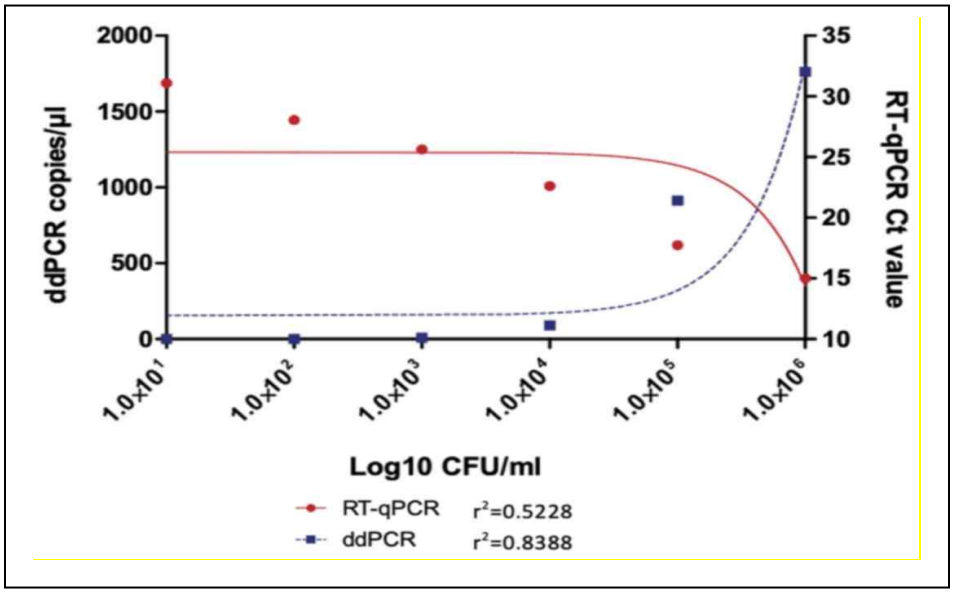

Figure 8.ddPCR and RT-qPCR linéar regression analysis of *L.pneumophila* dilutions (Falzone *et al.*, 2020)

4.2.1.2. The efficacy of thermal shock in ddPCR and RT-qPCR

Although ddPCR and RT-qPCR showed similar sensitivity in the detection of *L. pneumophila*, the simulation of heat shock treatment in an *in vitro* contaminated water tank highlighted the important differences existing between methods. (Falzone *et al.*, 2020)

RT-qPCR detected false-positive signals in the sample treated at 80°C for three days probably due to cell debris and residual degraded DNA that produced a nonspecific amplification signal. In addition, no significant variation was observed between the untreated samples after one day and three days of growth (Ct values of 22.00 and 23.06, respectively) (Falzone *et al.*, 2020) **(Table 4)**

Table 4.RT-qPCR Ct values before and after heat shock treatement (Falzone *et al*., 2020)

Number of samples	Sample	RT-qPCR Ct
1	10^2 CFU/ml day 1 no shock	29.72
2	10^2 CFU/ml day 3 no shock	28.51
3	10^2 CFU/ml day 3 post shock	-
4	10^2 CFU/ml day 5 no shock	25.35
5	10^2 CFU/ml day 5 post shock	35.18
6	10^2 CFU/ml day 7 post shock	-
7	NTC	-

Ct : Cycle threshold , **RT-qPCR** : Reverse transcription-quantitative polymerase chain reaction, **NTC**: No template control

On the contrary, ddPCR effectively identify as positive all the untreated samples and as negative the samples shocked at 80°C both after one day and three days of treatment. Moreover, ddPCR finely detected slight variation in the number of *L. pneumophila* after one day, three days and five days of growth without thermal treatments. In particular, after three days of incubation the number of copies increased from 1.9 to 5.3 copies/μl, while after 5 days of incubation the concentration increased at 25.5 copies/μl thus passing from an initial concentration of 10^2 CFU/ml to a concentration of 2.7×10^3 CFU/ml .

Of note, no nonspecific signals were observed for the sample treated at 80°C for three days, suggesting that ddPCR is less prone to interference from degraded DNA or cellular debris (Falzone *et al*., 2020)

4.2.2.Discussion

Different studies have demonstrated the higher sensitivity of ddPCR compared to RT-qPCR . (Hayden *et al* ., 2013 ; Arvia *et al* ., 2017)

At present, ddPCR is one of the most sensitive methods used for the detection of low amounts of targets, including circulating DNA, circulating mutations, rare copy number

variants and low viral nucleic acid targets representing a promising technology for use in clinical practice and environmental health . (Falzone *et al* ., 2020 ; Filetti *et al* ., 2020)

Several studies have tried to propose and validate RT-qPCR-based molecular methods for the detection of *L. pneumophila* in contaminated water samples but the sensitivity of the technique and the presence of inhibitor or contaminants may produce false-positive and false-negative results .(Lee et Lee , 2013 ; Shen *et al* ., 2015)

The results here obtained demonstrated that both ddPCR and RT-qPCR have a good sensitivity, however, high-sensitive RT-qPCR detected low concentration of *L. pneumophila* at a very late Ct value (Ct 31.10 for 10 CFU/ml concentration). On the contrary, ddPCR accurately identified low concentrations of *L. pneumophila* allowing absolute quantification of the bacterial load. (Falzone *et al* ., 2020)

These results also suggest that ddPCR may be used for the frequent monitoring of water samples before and after clean-up treatments in order to detect early *L. pneumophila* growth without waiting the long time necessary for the culture-based methods. (Falzone *et al* ., 2020)

It is known that in environmental samples there are several bacteria which together with cellular debris and degraded DNA can interfere with the correct detection of *Legionella*. However, although this represents a limit for culture methods and for molecular methods based on RT-qPCR, here we demonstrated that ddPCR is not affected by the presence of fragmented DNA or cell debris thanks to the nanopartitions of gene targets and the dilutions of contaminants into thousands of droplets. In this context, other studies support our findings and the use of ddPCR for the detection of bacterial DNAemia during infection or for the monitoring of bacterial load in contaminated samples with PCR inhibitors . (Singh *et al* ., 2017 ; Ziegler *et al* ., 2019)

Toplitsch *et al.* (2018) investigated the feasibility of qPCR for the detection of *L. pneumophila* in environmental water samples. This method relies on the quantification of *Legionella* DNA . (Toplitsch *et al* ., 2018 ; Saad *et al .,* 2020)

In the case of *Legionella* outbreaks, qPCR has drbastic benefits for the public health: samples can be tested with a high reproducibility, high sample throughput and a high specificity in a short time which allows to give out public health warnings faster**.** It is based on the simultaneous amplification of a nucleic acid target sequence, which can be calculated

back to reach the amount of genomic units (GU) per liter . This test could be used as a method for screening out negative samples in as quick as half a day after receipt of the sample in the laboratory and qPCR negative samples would then not go into the culture for testing . **(**Whiley et Taylor , 2016 ; Collins *et al* ., 2017 ; Toplitsch *et al* ., 2018)

The major advantages of qPCR in comparison with conventional culture method is low limit of detection, as well as the ability to detect VBNC cells . When used in conjunction with the culture method, qPCR can serve as a powerful tool. (Taylor *et al* ., 2014 ; Díaz-Flores *et al* ., 2015)

There are several drawbacks : qPCR typically overestimates *L.pneumophila* burden because it detects dead cells and the presence of PCR inhibitors may limit the use of this method. (Taylor *et al* ., 2014 ; Díaz-Flores *et al* ., 2015)

In addition, multiple processing steps are required which increases the overall cost of the qPCR method . (Whiley et Taylor , 2016)

Reuter *et al.* (2019) integrated the fast isothermal amplification method "Loop-mediated isothermal amplification" (LAMP) for amplification and quantification of *L. spp* and *L. pneumophila* with the colorimetric detection method using the metal indicator hydroxynaphtol blue (HBN) that enables a naked eye detection of the result (Blue-LAMP) in water samples.

The LAMP requires less sophisticated instrumentation (no temperature cycling) as well as lower maximum temperatures about 60 °C, and is more robust towards inhibitors.It can achieve excellent specificity, six primers targeting different loci of the target gene; its specificity in DNA amplification is therefore outperforming PCR and nucleic acid sequence based amplification (NASBA) and takes a lower reaction time < 1 h . (Reuter *et al* ., 2019)

In particular, the LAMP does not require an additional ligation process before amplification such as the rolling circle amplification (RCA) or a number of enzymes like recombinase polymerase amplification (RPA) and helicase-dependent amplification (HDA) . (Reuter *et al* ., 2019)

Another advantage is a straightforward product detection based on the large amounts of byproduct pyrophosphate that is produced during the reaction and reacts with Mg^{2+}; this reaction can be easily utilized for a simple colorimetric detection . The LAMP is based on 6

(or 8) distinct regions of the target sequence, which are used to design 4 (or 6) different primers and also eliminate the need to open the samples to add the dye reducing the risk of cross-contamination during amplification and detection of amplified DNA . (Reuter *et al* ., 2019)

In the range from 55 to 70 °C, the Blue LAMP reactions were positive with an optimum efficiency at 65 °C. This indicates that the developed LAMP assay is less sensitive to temperature fluctuations . Also Time requirements for performing the assay, from sample to results can be reduced to 1.5 h . (Reuter *et al* ., 2019)

This developed assay is suitable for quantification, but require additional detection instruments such as turbidimeters or fluorescence readers. (Reuter *et al* ., 2019)

There are three basic limitations of PCR-based detection assays are known, the first being the possible presence of PCR inhibitors that leads to false-negative results. The second is the advanced instrumentation required to ensure the right thermal cycling conditions, while both disadvantages can easily be bypassed through the use of the LAMP. The last limitation in terms of all DNA-based detection methods relates to their inability to distinguish between living and dead cells. DNA can persist for long periods after cell death that can lead to an overestimation of the actual risk. (Reuter *et al* ., 2019) .

Füchslin *et al.* (2010) presented a cultivation-independent, quantitative, and fast detection method for *L. pneumophila* in water samples. It consists of four steps, starting with a concentrating step, in which cells present in one litre of water are concentrated into 5 ml by filtration (pore size 0.45 µm), then cells are resuspended with sterile filtered buffer and double-stained with Alexa-conjugated *Legionella*-specific antibodies . Subsequently, the cells are immunomagnetically caught, and finally, fluorescently labeled *Legionella* cells were flow cytometrically detected and quantified. The efficiency of each step was tested separately. The whole method allows detection of *L. pneumophila* in 180 min with a detection limit of around 500 cells/l and a recovery of *Legionella* cells of 52.1 % out of spiked tap water**.**

This method does not allow the detection of *Legionella* cells that are integrated in a biofilm or located within amebae , and it can’t distinguish between living and dead cells. However, the smooth immunomagnetic separation enables that the detection can be coupled with the determination of the physiological state of the cells, for example: by life-dead

staining ; the BacLight bacterial viability kit or the carboxyfluorescein diacetate assay . That flow cytometry in combination with immunomagnetic separation will be an interesting alternative to standard plating methods in the near future. (Füchslin *et al .,* 2010)

Gruas *et al.* (2013) reported a comparative analysis of a number of environmental water samples using the ScanVIT-*Legionella*™ method and the traditional "gold standard "method of culturing and demonstrated the usefulness of the ScanVIT method.

ScanVIT- *Legionella*™ method based on gene probe technology enabling the quantification as well as the simultaneous detection of cultivable Legionella.spp and *L. pneumophila* within three days , All *Legionellae* colonies were seen as green micro-colonies while *L. pneumophila* colonies were visualised as red micro-colonies . This could be an additional advantage when rapid test results of water samples are needed, as during an outbreak or to evaluate the efficacy of disinfection interventions .This test can be used in laboratories where staffs are not experienced in identifying typical micro-colonies of *Legionella* . It's also offers a series of advantages such as quickly diagnosis and higher sensitivity . (Gruas *et al .,* 2013)

Among the disadvantages of the ScanVIT test is its inability to recover colonies from the filter for typing or biomolecular analysis, which are essential for the epidemiological correlation of human cases and environmental colonization. (Gruas *et al .,* 2013)

Meneghello *et al.* (2017) optimised the surface plasmon resonance(SPR) based device and applied for *L. pneumophila* detection strategy based on the highly sensitive azimuthally-controlled grating-coupling SPR (GC-SPR) technology . GC-SPR under azimuthal control demonstrated its ability in detecting specifically down to 10 CFU of *L.pneumophila* , a concentration beyond the Italian legal limit for high risk hospital environment, resulting a valid technology with a detection sensitivity up to 1000 folds higher than fluorescence assays here adopted as validation technology. The developed detection system represents the proof of concept of a novel *L. pneumophila* sensing technology in which the plasmonic platform could be easily integrated in a microfluidic sensor chip that could finally be used also by non highly-specialised personnel .

In order to control colonization by *L.pneumophila* , Párraga-Niño *et al.* (2018) developed a membrane filter method to capture and immunodetect this microorganism in

water samples. This membrane filter is used to retain the bacteria using a nitrocellulose discinside a home-made cartridge. Subsequently , they perform the immunodetection of the bacteria retained in the nitrocellulose (blocking, antibody incubation, washings and developing). And comparing this test with the gold-standard, the most important finding is the considerably reduction of detection time from 10 days to 2 hours with maintaining the same detection limit. The assay was able to detect 70 CFU of *L. pneumophila* in a 25 mm filter which make it capable of filtering volumes of 1 L . This rapid test is easily automated for *L. pneumophila* detection allowing a comprehensive surveillance of *L. pneumophila* in water facilities and reducing the variability in the analyses due to the low need for manipulation. The new protocol is economic since the hybridoma is avaible (monoclonal antibody) and simple , also it's ablility to detect viable and dead cells . Moreover, corrective measures may be applied the same day of the analysis .

Ezenarro *et al.* (2020) presented a combination of sample concentration, immunoassay detection and measurement by chronoamperometry. A nitrocellulose microfiltration membrane is used as support for both the water sample concentration and the *Legionella* immunodetection giving the chance to treat large sample volumes.

The horseradish peroxidase enzymatic label of the antibodies permits using the redox substrate 3,3',5,5'-Tetramethylbenzidine (TMB) to generate current changes proportional to the bacterial concentration present in drinking water .(Ahmed *et al .,* 2014) .

Carbon screen printed electrodes are employed in the chronoamperometric measurements. And as cells are retained in the membrane due to the filtration step, there is no need for a capture antibody. Thus, a single antibody system is used for the immunoassay which lowers the cost for each test . This system reduces the detection time from the 10 days required by the conventional culture-based methods to 2–3 h which could be crucial to avoid outbreaks and also could be integrated on the holder used for concentration. Additionally, the system shows a linear response (R2 value of 0.99), being able to detect a low concentrations of *Legionella* with range between 101 and 104 cfu·mL−1 , and a detection limit of 4 cfu·mL−1 . (Ezenarro *et al .*, 2020)

Ezenarro *et al.* (2020) accomplished the objective of obtaining a rapid ; economical and user-friendly system for *L.pneumophila* detection .

Moreover , The system was specially designed in such away that in future versions all steps can be automated and carried out by micropumps without the need for qualified personnel and fabricated with low-cost materials that could easily be mass-produce. (Ezenarro *et al* ., 2020)

Saad *et al.* (2020) reported a Systemic Evolution of Ligands through EXponential enrichment (SELEX) , it was used to identify aptamers which are short oligonucleotide sequence folding into a specific structure and are able to bind to specific molecules , in this study specifically to *L.pneumophila*. Two aptamers binding strongly to *L. pneumophila* were identified with K-dimensions (K_D) of 116 nM for R10C5 and 135 nM for R10C1 . Whereas R10C5 seems to stain *L.pneumophila* more strongly than R10C1, the latter seems more specific to *L.pneumophila* .

Binding specificity of these two aptamers to *L. pneumophila* was confirmed by flow cytometry and fluorescence microscopy. Therefore, these two aptamers are promising biorecognition molecules for the detection of *L.pneumophila* in real time and in situ . (Saad *et al .,* 2020) **(Appendice 03)**

4.3.Detection of *V. cholerae* by hybridoma

4.3.1.Results

4.3.1.1. Calcium measurement using fura 2-AM

A significant increase in intracellular calcium was observed after specific antibody-antigen binding with the sensitive fluorescent calcium indicator dye, fura 2-AM . And the spectral characteristics of fura 2-AM in hybridoma cell line are depicted in **Fig.09**. (Zamani *et al*., 2016)

The fluorescence emission spectrum showed a maximum intensity at 505 nm in the presence of *V. cholerae* O1, which was measured by excitation at 340 nm. Also, an obvious shifting in fura 2-AM excitation spectrum to shorter wavelengths was recorded after the addition of *V. cholerae* O1. The assay was performed in the same condition with no bacteria as control. (Zamani *et al*., 2016)

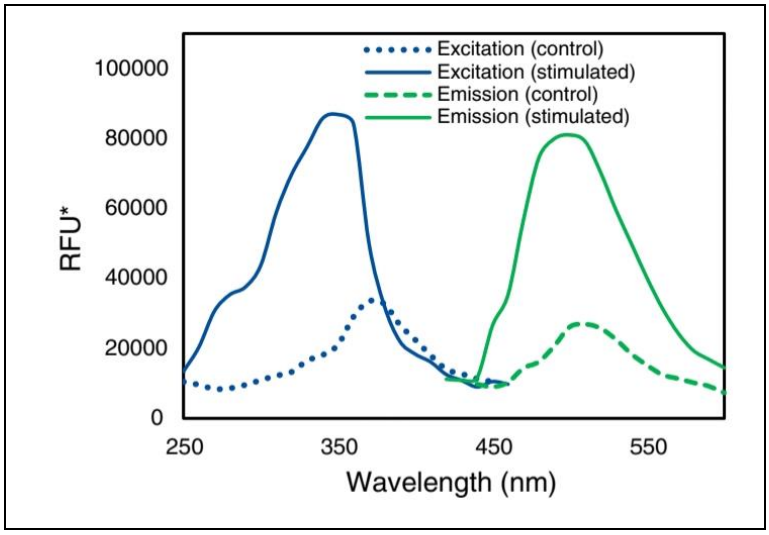

Figure 9. Spectral characteristics of fura 2-AM in hybridoma cell line. (Zamani *et al*., 2016)

4.3.1.2.Appropriate cell number per assay

Appropriate cell number, which influences the efficiency of the assay, was determined by loading various numbers of hybridoma cells with fura 2-AM and exposing to 200 CFU/mL of *V. cholerae* O1, which induced a rise in the intracellular free Ca^{2+} concentration. As shown in **Fig 10**, a significant rise in calcium concentration was observed in the presence of 5 × 104 *Vibrio*-specific hybridoma cells. The maximum efficiency of assay for detecting *V. cholerae* O1 was obtained at 2 × 105 cells and no significant difference in fura 2-AM emission was recorded in >2 × 105 cells. (Zamani *et al*., 2016)

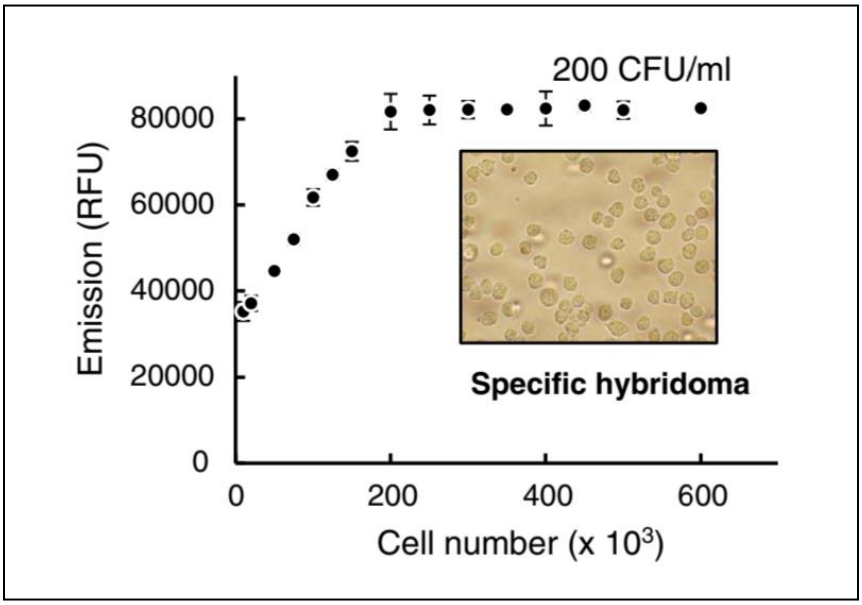

Figure 10.Appropriate cell number per assays. (Zamani *et al*., 2016)

In **Fig 10**. two hundred CFU/ml of *V. cholerae* O1 was exposed to various numbers of specific hybridoma cells and fura 2 fluorescence emissions at 505 nm were recorded.

4.3.1.3.Dose response curve and limit of detection

The assay sensitivity was investigated via addition of 2 × 105 specific hybridoma cells in the assay plate, followed by injection of *V. cholerae* O1 from 0 to 500 CFU/mL. It was seen that by increasing CFU, the fura 2-AM emission at 505 nm rose (Zamani *et al*., 2016)

4.3.1.4.Response time to *V. cholerae* O1

After exposing antigen to particular antibody, the fura 2- AM emission was recorded for 10 min and a significant increase in fluorescence emission started about 24 s after injection of 200 CFU/mL *V. cholerae* O1 to specific hybridoma cell.

And after 45 s : the maximum emission occurred which means this method can detect this bacterium in less than a minute demonstrating its potential to rapid screening of *V. Cholere* O1. (Zamani *et al*., 2016)

In addition, both time course and dose response plots confirmed that the maximum emission was observed after injection of 170 CFU/ mL (42.5 CFU per assay) in less than 45 s.

Also, as few as 50 CFU/ mL (<13 CFU per assay) was detected after several minutes by this method (Zamani *et al*., 2016)

4.3.1.5.Assay specificity to *V. cholerae* O1

The selectivity of this method for *V. cholerae* O1 detection was determined with two separate assays:

One was performed by exposing different bacteria (2000 CFU/mL) to *Vibrio*-specific hybridoma cell. (Zamani *et al*., 2016)

The second was done using different cell lines, including SP2/0 cell line and MC2B8 hybridoma cells with *V. cholerae* O1. (Zamani *et al*., 2016)

Fluorescence emission of fura 2-AM in the presence of *V. cholerae* O1 was significantly different from other pathogens .The same result was observed when *V. cholerae* O1 was added to other cell lines, including SP2 cells and MC2B8 hybridoma cell line. All data showed a significant difference between our current hybridoma and control samples. No significant responses to *V. cholerae* O1 were observed with other cell lines, whereas increased calcium concentration was recorded with *V. cholerae* specific hybridoma (Zamani *et al*., 2016)

4.3.1.6.Efficiency of the method in spiked environmental samples

The detection of *V. cholerae* O1 in different environmental samples was investigated and this assay was successfully used for detecting *V. cholerae* O1 directly from stool specimen, and the sensitivity of the assay was estimated about 55 CFU/mL.

The detection limit of the assay was computed to be 52 CFU/mL in environmental water, and there was no significant difference in the obtained sensitivity of water samples.

Also, maximum fluorescence emission was observed when samples were spiked with 170, 174, and 177 CFU/mL in fresh water, sea water, and stool sample, respectively. (Zamani *et al*., 2016)

4.3.2.Discussion

Until now, numerous specific hybridoma cells were produced by many laboratories for desired antibody isolation, and hybridoma banks supply research investigators worldwide with low cost monoclonal antibodies for studies in developmental and cell biology. In this study, Zamani *et al.* (2016) reveal a method for immediate detection of *V. Cholera* with specific available hybridoma. Time-consuming, difficult steps of B cell manipulation would be avoided by using whole hybridoma cells. Also, this method is ready to use and no special

training is required to utilize it. To date, detection of *V. cholerae* is typically carried out with various experimental procedures, including biochemical tests, molecular methods including PCR and multiplex PCR , LAMP **(Appendice 02)** , immunoassay using specific monoclonal antibodies , an immunofluorescent-aggregation (IFAG) assay, fluorescence in situ hybridization (FISH), and the direct fluorescent antibody assay (DFA) , a combination of culture methods, multiplex-PCR, and DFA , catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH), dipstick test, intrinsic catalytic activity of a magnetic polymeric nanoparticle, and application of biocompatible capped iron oxide nanoparticle. However, many of these methods currently used for *V. cholerae* detection are timeconsuming and most of them are not able to identify low numbers of the target organism. Vast arrays of genes, including zot, wbe, ace, tcpA, ctxA, ompW, ctxA, ctxB, and rfb have been used in PCR, which is a common method for *V. cholerae* O1 detection . In this research, Zamani *et al.* (2016) have developed a sensitive and specific method for effective detection of *V. cholerae* O1. For calcium measurement, hybridoma cell line was exposed to fura 2-AM, which showed high emission in 505 nm because of intracellular calcium releasing as a result of antigen binding to specific antibody .In this study, the pattern of elevation in intracellular calcium reported by a shift in the fura 2-AM excitation spectrum to shorter wavelengths is stated as 340/380 ratio . The sensitivity of the assay was evaluated using an increasing number of cells per assay to 2 × 105 cells/well in a 96-well plate.

The detection limit of the assay was determined to be 50 CFU/mL from pure culture and 50 % of maximal response was exhibited in the presence of 83 CFU/mL. Increase in 340/380 ratio was detected in the presence of *V. cholerae*, especially after the addition of >100 CFU/mL of this bacterium. Finally, this assay was successfully applied for *V. cholerae* detection in environmental samples, and the detection limit values were estimated to be as few as 52 and 55 CFU/mL in spiked environmental water and stool sample, respectively. Detection of different *V. cholerae* serotypes, including *V. cholerae* O1, in environmental samples was previously limited by laboratory testing, particularly PCR-based method, which led to time-consuming process involving DNA purification prior to PCR analysis to remove inhibitors . (Zamani *et al* ., 2016)

According to the results presented in the study of Zamani *et al.* (2016) a specific hybridoma cell enables us to detect O1 serotype of *V. Cholera* using a calcium indicator fluorescence probe. This method is designed for immediate detection of this bacterium with no additional process typically used for sensing system construction.

The sensitivity and specificity of this method demonstrate that specific hybridoma could be used as a sensitive and reliable diagnostic tool for other antigens, pathogens, and analyses in a broad spectrum of applications, including medical, environmental, and microbiological purposes. (Zamani *et al* ., 2016)

This method has advantages such as simplicity, ease of process, and it does not require manipulation of hybridoma cell. For this approach, an efficient amount of fluorescence Calcium indicator, fura 2-AM, was utilized, which emitted light when the intracellular calcium concentration increased as result of antigen binding to specific antibody. More reliable results are obtained via this method and it is considerably faster than other methods, which has the response time of less than 45 s for detection of *V. Cholerae* O1. Also, no significant responses were observed in the presence of other bacteria with specific hybridoma or other cell lines exposed to *V. cholerae* O1. Furthermore, this method was successfully applied to *V. cholerae* O1 detection in spiked environmental samples, including water and stool samples without any pretreatment. All results reveal that hybridoma cells can provide a valuable, simple, and ready to use tool for rapid detection of other pathogenic bacteria, toxins, and analyses. (Zamani *et al* ., 2016)

The fluorescent labelling technique uses antibodies to specifically identify pathogens, Wang *et al*. (2010) have applied this method to detection of *V. cholerae* in environmental water samples. established a fluorescent-antibody (FA) direct viable count (FA-DVC) assay which yields relatively high specificity and sensitivity and can rapidly and easily detect culturable and nonculturable *V. cholerae* O1. The direct fluorescent antibody (DFA) assay has also been applied to detect viable but nonculturable *V. cholerae* O1 in environmental water and in studies on the role of *V. Cholera* biofilm on the transmission of cholera. In addition, it developed two monoclonal antibody-based methods, the coagglutination test and the direct fluorescent-antibody test, which has a high specificity, and the detection limit for *V. cholerae* O139 in environmental water samples reached 2.0 × 103 CFU/ml and 1.5 × 102 CFU/ml, respectively.

Wang *et al*.(2010) also evaluated the feasibility of our IFAG assay by comparison with the direct enrichment, isolation, and culture method, as well as real-time PCR detection. The combination of IFAG and real-time PCR for environmental monitoring of *V. cholerae* can provide rapid and efficient detection of *V. cholerae* O1 and O139.

The sensitivity of the established IFAG method for detection of *V. cholerae* O1 and O139 in laboratory culture is 103 CFU/ml; the method is also highly specific. In the mixed bacterial

solution containing 10 species of interfering bacteria and the target *V. cholerae* O1 and O139 strains, IFAG is able to specifically detect fluorescent aggregates of *V. cholerae* O1 and O139 without cross-reactivity. Furthermore, the aggregates were verified to be *V. cholerae* O1 and O139 in the subsequent culture and serum agglutination tests, showing high specificity.

In the study of Herfehdoost *et al* .(2014) was to develop a new method for effective detection of *V. cholerae* O1.Which is based for nanoparticles for identification of bacterial DNA by PCR Dynabead. It use the biotinalated Probe for binding to DNA extracted from *V. Cholerae* and other bacterial species (*Salmonella, Shigella, Pseudomonas, E.coli*) Using magnetic bead isolated with magnetic field, the *Vibrio* genome-specific primers (HlyA) for pathogen detection (PCR) was used. The results showed specific band was just for *V. Cholera* (PCR positive); therefore designed probe was specific for *V. Cholerae*. According to the findings, this study is characterized the high sensitivity of PCR using biotin-containing probes for DNA of *V. Cholera* in contrast to the Traditional methods.

The purpose of this study Herfehdoost *et al*. (2014) was to develop a new method to isolate DNA target of *V. Cholerae* from other nucleic acids. Therefore, a capture probe specific for *Vibrio* that was immobilized on magnetic beads and used for DNA extraction followed by PCR. Polymerase chain reaction is extensively used to aid (and replace) traditional microbiology, as it allows for rapid identification of bacterial species and detection of virulence genes. The result of PCR based on nanotechnology in compare with other methods, indicated more specialty and sensitivity and in term of time and coast is affordable and the earned results in diagnostic and detection are reliable.

Huy *et al*. (2015) for the first time, protein A conjugated with chitosan-coated iron oxide nanoparticles was prepared for pathogen separation at low concentrations from liquid samples. *Vibrio cholerae* O1 (VO1) bacteria were used for testing the effectiveness of this conjugate. Transmission electron microscopy (TEM) was used to confirm the presence of captured VO1.

In the study of Huy *et al*.(2015) , a conjugate of protein A and chitosan-coated magnetic nanoparticles was prepared for separation of *V. cholerae* bacteria at low concentrations from water samples. Chitosan, a polysaccharide obtained from the N-deacetylation of chitin, is one of the most abundant polysaccharides in nature. It has been used for a wide range of biological applications, thanks to its biocompatibility and biodegradability. The results of the

study showed by TEM observation that the conjugate could easily separate *V. cholerae* bacteria from water samples at concentrations as low as 10CFU mL^{-1}. The selective separation of specific pathogens from liquid samples is achieved by incubation of the conjugate with a specific monoclonal or polyclonal antibody. The pathogens separated from a large volume of liquid samples can then be detected simply by conventional diagnostic methods or immunochromatographic strip tests on-site. More importantly, it has the potential to be used for the separation and detection of even unknown pathogens in clinical samples collected from emerging outbreaks.

Zamani *et al*. (2016) describes the development of a simple, sensitive and specific method for detection of *V. cholerae* using hybridoma cells containing antibody against cholerae O1 antigen instead of engineered B cells used in Cellular Analysis and Notification of Antigen Risks and Yields (CANAR) technology. Although there are many published methods, such as those mentioned above, the trend has become towards the development of faster detection assay distinguishing a few CFU of this bacteria. hybridoma cell selected in this study potentially enables detection of *V. cholerae* O1 serotype. The results revealed that the hybridoma cell is a suitable candidate for specific sensing element in biosensor construction and it can be utilized in other diagnostic tools. Also, the speed, sensitivity and specificity of constructed biosensor are valuable attributes for *V. cholerae* detection and it can be considered as a fast and reliable diagnostic tool for other pathogens, toxins and environmental compounds in different applications.

Hao *et al*. (2017) used Point of care tests (POCT) method called UPT-LF **(Appendice 01)**, which is an up-converting phosphor technology-based lateral flow (UPT-LF) assay with a dual-target detection mode, was developed to detect *V. cholerae* O1 and O139 simultaneously from one sample loading. Although applying an independent reaction pair made both detection results for the two UPT-LF detection channels more stable, the sensitivity slightly declined from 104 to 105 colony-forming units (CFU) mL^{-1} compared with that of the single-target assay, while the quantification ranges covering four orders of magnitude were maintained. The strip showed excellent specificity for seven *Vibrio* species that are highly related genetically, and nine food-borne species whose transmission routes are similar to those of *V. cholerae*. Therefore, successful development of UPT-LF as a dual-target assay for quantitative detection makes this assay a good candidate POCT method for the detection and surveillance of epidemic cholera. Field evaluation of water samples

demonstrated that UPT-LF is as sensitive as real-time fluorescent PCR with a lower false positive rate under pre-incubation conditions, implying it is a candidate POCT method for use in a surveillance system for the prevention and control of epidemic cholera. For detecting *Vibrio cholerae*, a new chromogenic medium by Briquaire *et al*. (2017**)** was designed and evaluated as an alternative to thiosulfate citrate bile salts sucrose (TCBS) agar for testing raw water samples. Sensitivity and specificity of the medium were assessed using both raw and spiked water samples. The *V. cholerae* chromogenic medium was proved to be highly selective against most of the cultivable bacteria in the water samples, without loss of sensitivity in detection of *V. cholerae*.

Thus, reliability of this new culture medium for detection of *V. cholerae* in the presence of other *Vibrio* species in water samples offers a significant advantage. The paper based analytical device (PAD) provides advantages in that standard culture methods employing agar plates are not required. Also, intermediary isolation steps were not required, including transfer to selective growth media, hence these steps being omitted reduced time to results. Furthermore, experienced technical skills also were not required. Thus, PAD is well suited for resource-limited settings. reliability of this new culture medium for detection of *V. cholerae* in the presence of other Vibrio species in water samples offers a significant advantage. The paper based device (PAD) containing the new chromogenic medium was proved to be a useful test for *V. cholerae* in water supplies in remote settings, especially resource poor countries. PAD provides a low cost, rapid, reliable test for *V. cholerae* detection.

Rashid *et al*. (2017) developed a method of detection of *V. Cholera* which is the Crystal VCâdipstick test had a high specificity (>99%) relative to culture for measuring *V. cholerae* in both stored household drinking water and municipal sup-ply water from cholera patient households .However, its sensitivity was lower than expected at only 65%. The performance of the dipstick test was similar for both household stored and municipal supply water sources. the Crystal VCâdipstick test had a much lower sensitivity than previously reported .

In the rapid diagnosis, usage of the terms "sensitivity" and "specificity" make confusion in the diagnostic use of test results. In most of the investigations discussed in this article covers the above two aspects. These two terms are distinct from that assay's clinical diagnostic sensitivity and diagnostic specificity. "Diagnostic sensitivity" is the percentage of individuals who have an infectious condition and identified by the assay as positive. It must

be noted that high analytical sensitivity does not ensure acceptable diagnostic sensitivity. "Diagnostic specificity" is the percentage of individuals who do not have an infectious condition as identified by the assay as negative. Validated assay systems with high sensitivity and specificity are important for the rapid identification of cholera cases and asymptomatic carriage. Due to the lack of acceptable sensitivity (≥90%; the Rapid diagnostic tests (RDT) are not contemplated as a substitute for culture methods. In RDTs, the specificity and sensitivity remain less than the epidemiological optimal mark and therefore their use in diagnosis remains a big challenge. RDT can be considered as an investigative tool in cholera outbreak settings since the culture techniques are often difficult to establish due to the dearth of skilled manpower, media... etc.

(Ramamurthy *et al* ., 2020)

Conclusion

Conclusion

At the end of this study, we were able to compare the different methods of detecting pathogenic bacteria in water, and finally, after the discussion, we were able to identify the most effective methods in terms of detection speed, sensitivity and specificity.

About ***V.cholerae*** although signs and symptoms of acute cholera are evident in areas where infection is common, the only way to be sure of the diagnosis is to identify the bacteria in a stool sample. The most effective methods are PCR based on nanotechnology in compare with other methods, indicated more sensitivity and in term of time and coast is affordable and the earned results in diagnostic and detection are reliable. And the direct fluorescent antibody sensitivity and specificity 100% with 10 CFU/ml.

About ***E.coli*** the most effective method is portable culture device based on POC premises. This method can detect *E.coli* after few hours from incubation and the limit of detection is 1 CFU/mL.

Finely about ***L.pneumophila*** the most effective method is a membrane filter method to comparing this test with the gold-standard, the most important finding is the considerably reduction of detection time from 10 days to 2 hours with maintaining the same detection limit. The assay was able to detect 70 CFU of *L. pneumophila* in a 25 mm filter which make it capable of filtering volumes of 1 L.

In our selection of the most effective methods, we relied on several criteria, the most important of which is the time of detection, the lowest value of CFU that can be sensed, and the largest possible sample in which the pathogenic bacteria are detected.

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Web site

https://medicine.uiowa.edu/humangenetics/genomics-division/quantitative-dnarna-analysis/droplet-digital-pcr

Appendices

Appendices

Appendice 01

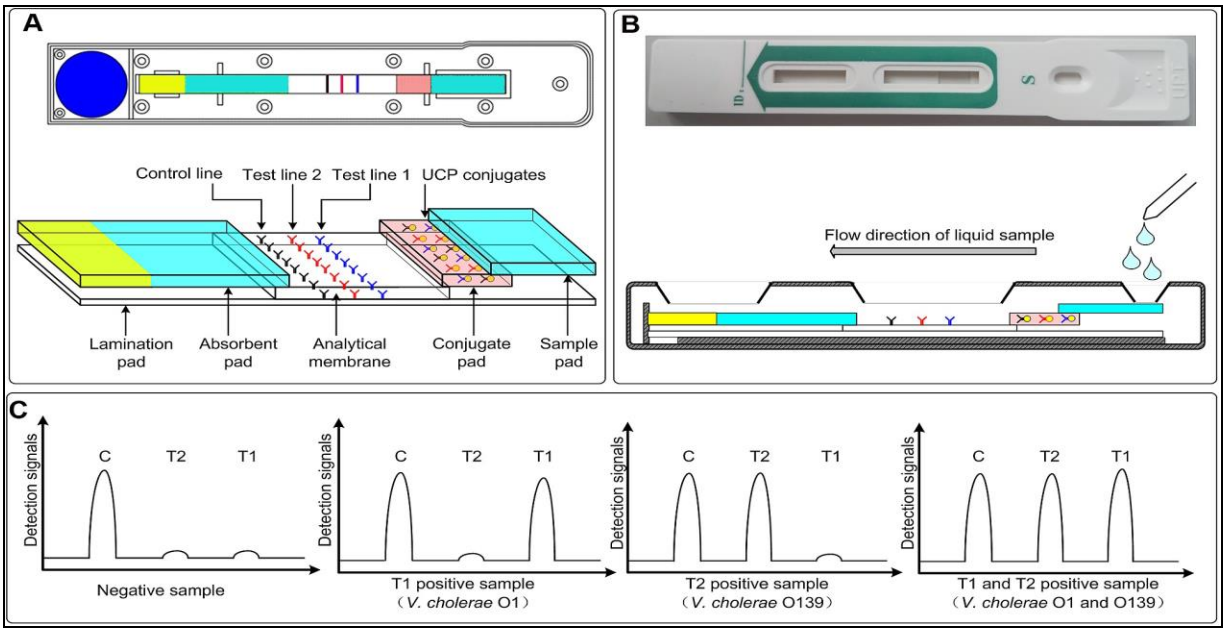

Figure1. Schematic diagram for the UPT-LF assay. (Hao *et al* ., 2017)

Appendice 02

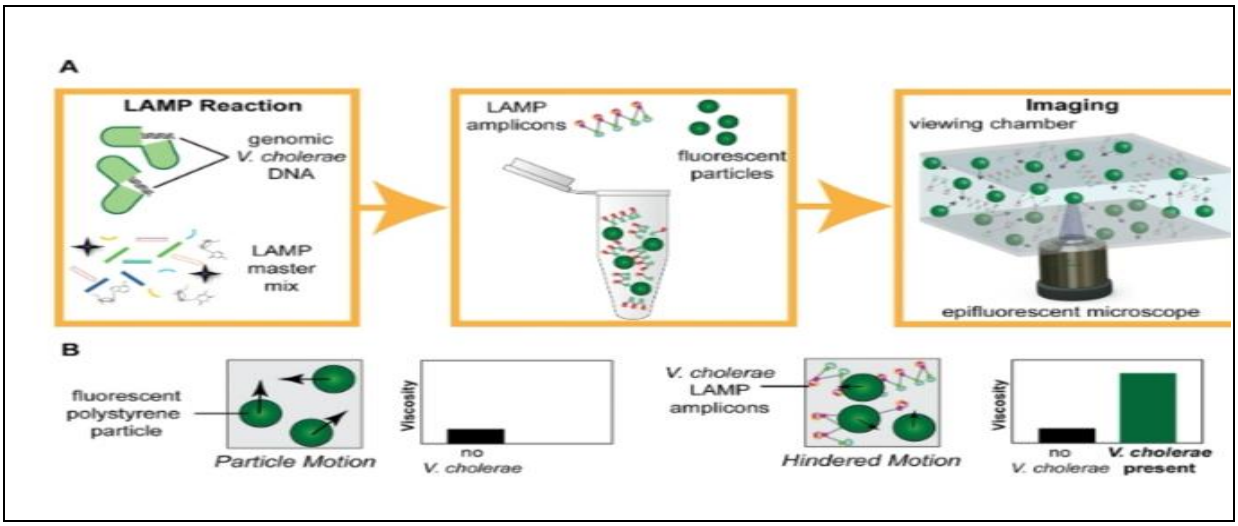

Figure 2. Illustration of LAMP set-up. (Clayton *et al* ., 2019)

Appendice 03

Table 1. Other techniques used for the detection of *Legionella* from water samples

(Walker et McDermott , 2021)

Test Method	**Plate culture**	**MPN**	**IMS**	**LF**
Time of results	7-14 days	7 days	Same day	Same day
Quantificatin	Yes (CFU/Volume)	Yes (MPN/Volume)	Yes (CFUeq/volume)	No
Liver or dead cells	Live	Live	Live and potentially dead/damaged cells	Live and potentially dead/damaged cells
Detect VBNC	No	No	Yes	Yes
Legionella.spp	Yes	No	Yes	-
Legionella Pneumophila	Yes	Yes	Yes	Yes
Isolate avaible	Yes	Yes	No	No
Sensivity	Low	98 %	95.3%	Unknown
Specificity	95.3%	> 97,9 %	88.4%	Unknown
False Positive	83%	< 4%	11.6%	Unknown
False Négative	74%	4,2%	4.7%	Unknown
Sample preparation	Yes	Only for non-potable samples	Yes	Yes
On-site test	No	Yes	Yes	Yes

Laboratory test	Yes	Yes	Yes	No
Routine monitoring	Routine	Routine	Routine	Routine
Specialist expertise required	Yes	No	Yes training provided	No
LOD	1 CFU/100 ml	>1 organism /100 mL	Equivalent to culture	100 CFU/L

MPN: Most Probable Number , **IMS:** Immunomagnetic Séparation , **LF:**Lateral Flow, **LOD:** Limit of Detection , **CFU:** Colony Forming Unit , **VBNC :** Viable but non-culturable

الملخص

يعتبر الماء من اهم العناصر الموجودة على سطح الارض و يتم استهلاكه باستمرار في الاكل و الشرب و الزراعة و الصناعة ، نظرا للاهمية الكبيرة للمياه فانه يجب مراقبة جودتها و الحرص على ان تكون خالية من جميع الملوثات بالاخص الميكروبات و ان تكون قابلة الاستهلاك وفق معايير محددة .

و قد تطرق هذا العمل الى جانبين الاول يتعلق بدراسة نظرية عن الماء و دورة حياته و مصادره ، كذلك خصائصه الفيزيائية و الكيميائية ، و قد أظهرنا خصائصه الميكروبيولوجية مع التركيز على البكتيريا الملوثة للماء التي تسبب أمراض خطيرة.

الجانب الثاني يتعلق بدراسة و تحليل بعض المقالات العلمية التي تناولت دراسة أحدث التقنيات المستخدمة في الكشف عن أهم الميكروبات الملوثة للماء المتمثلة في *E. Coli , L. Pneumophila , V.Cholerae* باستخدام هذه التقنيات على التوالي: اختبار الغمس ، تفاعل البوليميراز المتسلسل الرقمي القائم على القطيرات ، تقنية الورم الهجين .

في الاخير توصلنا كلما كانت مدة الكشف قصيرة و حد الكشف ضئيل ، كذلك زيادة حساسية و دقة التقنية هذا يؤدي الى الكشف السريع عن البكتيريا الممرضة و بالتالي اتخاذ الاجراءات اللازمة للتحكم في انتشارها و معالجة المرضى بسرعة.

الكلمات المفتاحية : ، *E. Coli* ، *L. Pneumophila* ، *V. Cholerae* ، اختبار الغمس ، تفاعل البلمرة المتسلسل الرقمي القائم على القطيرات ، تقنية الورم الهجين ، الكشف .

Résumé

L'eau est l'un des éléments les plus importants à la surface de la terre. Il est constamment consommé dans les aliments et les boissons, l'agriculture et l'industrie. En raison de la grande importance de l'eau, sa qualité doit être surveillée et elle doit être exempte de tous les polluants en particulier les microbes, et doit être consommée selondes normes spécifiques.

Ce travail comportait deux aspects. Le premier est lié à une étude théorique sur l'eau, son cycle de vie et ses sources, ainsi que ses propriétés physiques et chimiques. Nous avons montré ses propriétés microbiologiques en se basant sur les bactéries pathogènes d'origine hydrique qui causent des maladies dangereuses.

Le deuxième aspect concerne l'étude et l'analyse de certains articles scientifiques qui ont démontré les technologies récentes utilisées dans la détection du bactérie pathogènes le plus important représenté par : *E.coli ; L. Pneumophila, V.Cholerae* en utilisant respectivement ces techniques : test d'immersion, PCR numérique à gouttelettes, technologie des hybridomes.

Au final, nous avons conclu que chaque fois que la période de détection est courte et que la limite de détection est petite, ainsi que l'augmentation de la sensibilité et spécificité de la technique, cela conduit à la détection rapide des bactéries pathogènes, et ainsi à prendre les mesures nécessaires pour contrôler leur propagation et traiter les patients rapidement.

Abstract

Water is one of the most important elements on the surface of the earth. It is constantly consumed in food , drink, agriculture and industry. Due to the great importance of water, its quality must be monitored and it must be free from All pollutants especially microbes, and must be able to be consumed according to specific standards

This work included two aspects. The first is related to a theoretical study on water, its life cycle and its sources, as well as its physical and chemical properties. We have also shown its microbiological properties based on waterborn pathogen that cause dangerous diseases .

The second aspect relates to the study and analysis of some scientific articles that demonstrate the recent technologies used in the detection of the most important waterborn pathogene represented by: *E. Coli* ; *L .Pneumophila* ; *V.Cholerae* using these techniques respectively: Dip test, ddPCR, hybridoma technology.

In the end, we concluced that whenever the detection period is short and the detection limit is small, as well as the increase in the sensitivity and spécificity of the technique. This leads to the rapid detection of pathogenic bacteria and thus taking the necessary measures to control their spread and treat patients quickly