Study on Coliforms Detection in Water

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ABSTRACT

Coliforms have been used as indicators to denote water quality. Unpolluted environmental water usually does not contain high concentration of coliforms. Coliforms are found in large numbers in warm-blood animals' intestinal flora, and thus in their fecal wastes. An observation of coliform presence in the water sample indicates possible fecal contamination of the water source, consumption of which could cause severe waterborne disease outbreak. Coliform counts in water therefore, should be strictly controlled. Tests used to identify coliforms target at different characteristics, and the definition of coliforms varies with the detecting technique. The study is to review the current methods being applied in coliforms detection for water samples and to give a comprehensive understanding of these techniques.

BIOGRAPHICAL SKETCH

Rachel Yu is a MPS student in Agriculture and Life Science department in the field of Food Science, and expected to graduate at May 2019. She graduated from UC Davis with Bachelor of Science degree in Biomedical Engineering and Bachelor of Art degree in Comparative Literature in 2018. After joining Cornell at 2018, she has been researching on coliform related topics, especially on the filed that using coliforms as indicators for water quality.

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Thank you, Cornell. It has been an amazing year.

Rachel Yu

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Abstract

Coliforms have been used as indicators to denote water quality. Unpolluted environmental water usually does not contain high concentration of coliforms. Coliforms are found in large numbers in warm-blood animals' intestinal flora, and thus in their fecal wastes. An observation of coliform presence in the water sample indicates possible fecal contamination of the water source, consumption of which could cause severe waterborne disease outbreak. Coliform counts in water therefore, should be strictly controlled. Tests used to identify coliforms target at different characteristics, and the definition of coliforms varies with the detecting technique. The study is to review the current methods being applied in coliforms detection for water samples and to give a comprehensive understanding of these techniques.

1. Introduction

Coliforms have long been used as indicators to determine if a water source has been contaminated with potentially dangerous microorganisms. Coliforms are a classification of bacteria that is based upon their ability to ferment lactose. A subset of coliforms are found in high numbers in warm-blood animals' intestinal flora, and thus, in their fecal waste. They usually are not found in large numbers in unpolluted environmental water sources, which refers to any water in a river or wetland that found in nature [1]. Therefore, the presence of coliforms in a water sample suggests possible fecal contamination of the water source, consumption of which could cause severe waterborne disease outbreak and in some extreme cases death. Treated water that intended for drinking purpose should be coliform-free. Any positive total coliform samples in treated water could indicate ineffectiveness of the treatment [2], and should not be tolerated.

2. Regulations about Coliforms

The surveillance of drinking water quality is the responsibility of many associations and regulatory agencies including APHA (American Public Health Association), EPA (Environmental Protection Agency), and AOAC International (Association of Official Analytical Chemists/Communities). Each entity defines coliforms differently from one another based on different detecting methods. Three of these associations and regulatory organizations will be discussed and used as standards in this review: APHA, EPA, and AOAC; all of have defined coliforms on a different because of the different detection methods they endorse.

American Public Health Association is the largest association of public health professionals in the world; it is not a Federal agency. Since it was founded in 1872, APHA has been dedicated to improving the overall health of all residents of the United States [3], and has played a prominent advocacy role on many health related issues, such as assuring the availability of clean air and water, creating a safe and nutritious food supply, guiding people to adopt healthy lifestyles, monitoring the environment for adverse effects on human health, guaranteeing comprehensive and appropriate maternal and child health services, expediting the full immunization of the population against vaccine-preventable disease, and facilitating the development of safe work environments [3]. APHA has always been advocated the government to adopt the most current scientific advances that are relevant to public health, and naturally coliforms detection in water would be one of their concerns in order to promote general public health.

A federal agency that is responsible for monitoring the water supply is the Environmental Protection Agency (EPA), which is an independent agency of the United States federal

government charged with environmental protection. EPA enforces federal clean water and safe drinking water laws, provides support for municipal wastewater treatment plants, and takes part in pollution prevention efforts aimed at protecting watersheds and sources of drinking water [4]. The agency is responsible for water quality surveillance and response, and hence, coliforms detection in water is under EPA's regulation.

AOAC, which is not a regulatory, is an international non-profit scientific association that was found initially in 1884. It publishes standardized analysis methods designed to increase confidence in the results of chemical and microbiologic analysis. Government agencies and civil organizations often require that laboratories to use official AOAC methods, which exemplifies the high credibility of the association. An AOAC approved method, even not approved by regulatory agencies, should be efficient enough to detect targeted microorganisms, which in this case, the coliform group.

The definition of coliforms also varies with regions, countries and regulatory agencies, for example European countries has established their own system of identifying coliforms in water, and so does Canada; however, the differences and the definitions applied outside of United States will not be discussed in this review.

3. Definitions of Coliforms

The definition of coliforms varies with the methods used for their detection. APHA defines coliforms based on its biochemical characteristic that they ferment lactose. APHA endorses two detection methods, multiple-tube fermentation method and membrane filtration method. The *Standard Methods for the Examination of Water and Wastewater* defines the coliform group based on their ability to ferment lactose, where the two methods defines

coliforms differently due to different experimental procedures and results seen accordingly. With the multiple-tube fermentation technique, coliforms are defined as facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35°C [5]; while the membrane filter technique is applied, the coliform group is defined as those facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that develop red colonies with a metallic (golden-green) sheen within 24 h at 35°C on an Endo-type medium containing lactose [6]. The methods endorsed by the APHA also includes another definition of coliforms based on enzymatic activity using chromogenic substrates. Coliforms are defined as any bacteria possessing the enzyme β-D-galactosidase [7]. Commercially available tests Colilert and Colilert 18 that are developed based on the enzymatic activity have also been approved by APHA as standard tests for coliforms' detection.

EPA, on the other hand, has also approved several methods that reflect the quality of drinking water. EPA has included the multiple-tube fermentation technique, the membrane filter technique, the presence/absence test, which is a test developed based on the β-D-galactosidase activity of the coliforms, on its approved tests list; and the definitions of coliforms align with the ones that APHA has endorsed. Moreover, EPA has also approved membrane filtration test, which combines the membrane filter technique and enzymatic assay for faster coliforms detection in drinking water.

AOAC has approved not only all the methods that have been mentioned above [8], but also other methods that have been developed more recently. AOAC has approved a Petrifilm Aqua Coliform Count Plates developed by 3M in 2011. 3M Petrifilm Aqua Coliform Count Plate has been validated against membrane filtration method that published by APHA Standard Methods for the Examination of Water and Wastewater [9]. In 2015, AOAC has approved BAX

system PCR assay for *E. coli* O157:H7 [10], even though the system is mainly used in meat inspection, and has not been widely adopted by water quality surveillance system.

Researches have been reported on the development and validation of molecular methods, including methods based upon immunological and nucleic acid detection, for use in monitoring drinking water quality. Molecular methods could usually provide accurate results and allow direct recognition. However, despite of some prospective results that was generated by researches, none of the techniques have not yet been approved by any of the agencies mentioned above for monitoring water quality. But still, the molecular methods that are currently being developed are another main branch of methods for the identification of coliforms in water and are promising techniques that could be used to accurately detect coliforms.

4. Methodology

4.1. Culture-based Methods

4.1.1. Multiple-tube fermentation technique

In 1988, APHA endorsed the multiple-tube fermentation technique as standard test for the coliform group and the technique has been used as one of the standard methods where the results are often used as benchmarks to compare results from newly developed tests of coliforms detection. The multiple-tube fermentation technique is carried out by inoculating water samples in serial dilutions, and the fermentation of lactose is monitored. The technique is a two-phase test; a presumptive phase and a confirmation phase. When a positive presumptive test result is obtained, meaning the water sample contains microorganisms that can ferment lactose, a confirmation test is required to verify the positive result. In presumptive test, water samples are added to lauryl tryptose broth, which contains tryptose, lactose, dipotassium hydrogen phosphate

(K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), sodium lauryl sulfate and reagent-grate water, that has been refrigerated after sterilization [5]. After mixing the broth with water samples, bromocresol purple is added as the indicator to determine acid production, and hence, a positive result. A positive presumptive test is constituted when production of gas or acid formation in tubes within 48 $\pm$ 3 hours of inoculation at 35 °C. Tubes with a positive presumptive reaction require confirmation test to verify the result; tubes with a negative presumptive reaction may also be submitted to the confirmation stage to completely excludes some occasional members of the coliform group that grow very slowly and have not been detected in the previous phase. Usually, a confirmation of negative presumptive test is conducted very rarely and only on drinking water samples to ensure safe consumption. A confirmation test is conducted by first transferring the culture to a fermentation tube containing brilliant green lactose bile broth. The brilliant green lactose bile broth contains peptone, lactose, oxgall, brilliant green, and reagent-grade water [5]. The formation of gas in a brilliant green lactose bile broth fermentation tube at any time within 48 hours at 35°C constitutes a positive test for total coliform.

In order to distinguish the detected presumptive total coliforms are fecal coliforms, fecal coliform test using an EC medium is performed. An EC medium is composed of tryptose or trypticase, lactose, bile salts mixture of bile salt No. 3, dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), and reagent-grade water. Cultures from each presumptive fermentation tube would be transferred to EC broth and incubated in water bath at 44.5 $\pm$ 0.2 °C for 24 $\pm$ 2 h. Production of gas within 24 $\pm$ 2 hours or less at 44.5 °C in an EC broth is recognized as a positive test for fecal coliform.

The result of multiple-tube fermentation technique is expressed in the most probable number (MPN) of microorganisms present. This number, based on certain probability formulas, is an estimate of the mean density of coliforms in the sample. And based on coliforms density and other parameters, the sanitary quality of source water could be assessed. However, the method could only offer a semi-quantitative enumeration of coliforms; the precision of the estimation is low and it highly depends on the number of test tubes used for the analysis. Because bacteria in samples are randomly distributed in each tube, the more tubes used in the test would lower the chance of generating false negative results. This is to say that the more tubes used in the analysis, the higher sensitivity the test would be. When a 1 ml sample is examined containing 1 coliform/ml, about 37% of 1-ml tubes may be expected to yield negative results; however, if five tubes are used for the test, where each tube still only contain 1 ml sample, a negative result may be expected less than 1% of the time [11]. When choosing the number of tubes should be used for optimal results, APHA gives suggestion stating that the ideal number of dilutions should be reached when the largest sample inoculum examined shows gas in some or all of the tubes and the smallest sample inoculum shows no gas in all or a majority of the tubes [5]. AOAC refers multiple-tube fermentation method as 3-tube Most Probable Number (MPN) methods [4], which describe the same procedures and interpretation of the results.

Multiple-tube fermentation technique is easy to implement and requires only basic laboratory equipment. However, it is extremely time consuming: both the presumptive test and confirmation test take at least 48 hours of waiting time, so in order to get a confirmed result, 96 hours waiting time is necessary. Moreover, studies have shown a significant decrease in the specificity of presumptive test when high numbers of non-coliform bacteria present in water samples. Seidler et al showed that the presumptive test using lauryl tryptose broth failed to detect

coliforms in about one half of the coliform-contaminated positive presumptive tubes, where among 210 positive presumptive tubes, only 93 were confirmed to be coliforms, giving a 44% false positive rate of the presumptive test [12]. Significant coliform masking also occurred in the turbid (gas-negative) presumptive tubes in the same experiment, where coliforms could not be appropriately detected during the presumptive test phase. Among the 276 negative presumptive tubes, 26 of them were confirmed to be coliforms using brilliant green lactose bile broth, generating a 9.4% false negative rate [12]. Evans et al also found similar result implying the high false-negative rate of the presumptive test is related to the cloudiness of the samples, and based on their findings, 58% of the presumptive positive samples found to be false negative ones [13].

4.1.2. Membrane Filter Technique

Membrane filter technique is generally more accepted than multiple-tube fermentation technique mainly because it requires less time to result, and it could be used in testing larger sample volume than multiple-tube fermentation technique. It can be applied in drinking, surface, ground, swimming pool, marine, and diluted waste waters. It has also been widely used as parallel test to validate other coliform detecting tests in water samples. APHA, EPA and AOAC have all approved membrane filter technique as a standard test for water quality control. Like multiple-tube fermentation technique, membrane filter technique exploits the lactose fermenting characteristic to identify the coliform group.

Membrane filter technique utilizes sterile filters with 0.45um pore size to filter water samples and retain the bacteria. The amount of water sample should be predetermined based on the source to prevent resulting in crowded plate. In order to achieve the optimal estimation of coliforms, the suggested sample volume provided by APHA for drinking water when applying

membrane filter technique is about 100 ml, and for river water, the range is between 0.001 ml to 1 ml. The filter is then incubated on m-Endo-type media containing lactose for 24 hours at 35 °C. The m-Endo-type media contains tryptose or polypeptone, thiopeptone or thiotone, casitone or trypticase, yeast extract, lactose, sodium chloride (NaCl), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium lauryl sulfate, sodium desoxycholate, sodium sulfite (Na_2SO_3), basic fuchsin, and reagent-grade water. Coliform bacteria would form red colonies with a metallic (golden-green) sheen, and thus indicate a positive total coliform result. A fecal coliform test requires the filter to be incubated on an enriched lactose medium, mFC medium is often used, for 24 $\pm$ 2 hours at 44.5 $\pm$ 0.2 °C. mFC medium is composed of tryptose or biosate, proteose peptone No. 3 or polypeptone, yeast extract, sodium chloride (NaCl), lactose, bile slat No. 3 or bile salts mixture, aniline blue, and reagent-grade water. Colonies produced by fecal coliform bacteria on mFC medium appear various shades of blue, whereas nonfecal coliform colonies are gray to cream colored.

Some members of the total coliform group could produce dark red, mucoid, or nucleated colonies without a metallic sheen that is differently from what the definition has described, and occasionally, typical sheen colonies may be produced by non-coliform organisms. In both cases, verification is recommended by APHA to both identify the aberrant coliforms and to exclude non-coliform lactose-fermenters such as *Aeromonas* and *Pseudomonas*, which have also been important competitors to coliform bacteria growing on m-Endo media. Study showed that the false-positive rate of m-Endo medium when testing total coliforms is about 22%, while the false-positive rate of m-FC medium when testing on fecal coliforms is about 28% [14]. A lactose fermentation test is therefore, recommended as a means of verification, where each colony needs to be transferred to place in lauryl tryptose broth and incubate at 35 $\pm$ 0.2 °C for up to 48 hours.

Gas formation in lauryl tryptose broth and confirmation in brilliant green lactose broth within 48 hours verifies the colony as a coliform. During the verification process, simultaneous inoculation of both media (lauryl tryptose broth and brilliant green lactose broth) for gas production is acceptable. Incubation of EC broth inoculation at 44.5 $\pm$ 0.2 °C would confirm if fecal coliforms are present. Verification can also be done by using enzymatic properties of coliforms. A rapid verification of colonies utilizes test reactions for cytochrome oxidase (CO) and for β-galactosidase. Coliforms should generate CO negative and β-galactosidase positive results within 4 hours incubation of tube cultures.

The enumeration of coliforms is determined by the coliform colonies formed in the medium, and is expressed in colony-forming units (CFU). It is a more quantitative estimation comparing to the most probable number result that is used in multiple-tube fermentation technique. However, the count usually underestimates the number of viable coliform bacteria, since stressed and injured coliforms are incapable of recovering from the incubation, which causes a huge concern of this technique, especially when it is used on drinking water quality analysis.

Drinking water usually is treated with chemicals, for example chlorine, before it is delivered to consumers. The disinfection could cause sublethal injury to coliform bacteria, resulting in damaged cells that are unable to form a colony on a selective medium, however, they are still viable and could cause harm to the consumers. Improved new medium, m-T7 medium is designed to recover stressed coliforms in drinking water has been developed [16]. The new medium consists of Difco proteose peptone No. 3, lactose, yeast extract, Tergitol 7 (25% solution), polyoxyethylene ether W-1, bromthymol blue, bromocresol purple, and distilled water [16]. Coliform colonies appear as yellow colonies on m-T7 medium. m-T7 medium was able to

recover 86 to 99% more laboratory-injured coliforms than m-Endo agar could, and was capable of recovering about 43% more verified coliforms from surface and drinking water samples than m-Endo medium [11]. Especially in drinking water, m-T7 agar recovered nearly three times more coliforms than m-Endo membrane filter technique did. The sensitivity of the new medium is greater than 70%, and its false negative rate is lower than 0.5% [11] However, m-T7 medium only show such great recovery rate and sensitivity on chlorine treated water samples; when applied on monochloraminated [17] or ozone treated [18] water samples, m-T7 medium showed no significant difference with m-Endo medium.

Other studies have suggested that chemical treatment such as chlorination reduces catalase enzymatic activity, which affects the degradation of hydrogen peroxide (H_2O_2) produced in cells. The accumulation of toxic hydrogen peroxide would jeopardize the recovery of coliforms during incubation. By adding of catalase, sodium pyruvate or both into the media that has mentioned above for membrane filter technique, the true-positive rate in the confirmation stage on all media has shown either an increase or a comparable result: on an m-FC medium, 83% fecal coliforms recovery rate was observed with catalyst added and the control medium that contains no catalyst showed 77% recovery rate; on m-Endo medium, both control medium and catalyst medium showed 97% recovery rate; with catalyst addition, m-T7 medium showed 95% true positive rate, which is higher than 90% in the control medium [19]. The choice of medium could also significantly influence the rate of false positive and negative results. There is not a universal medium that could give the optimal results for water samples under all conditions. Designing media that best recover coliforms in water samples is a possible route to increase the test performance.

Membrane filter technique also shares similar limitations with multiple-tube fermentation technique. The results of both are usually interfered by background heterotrophic bacteria. Coliforms recovery rate would decrease when water samples contain high turbidity or large numbers of non-coliform bacteria [20]. Excessive crowding of colonies on m-Endo media has also been associated with a reduction in coliform colonies producing the metallic sheen. Experiment showed that up to 200 coliforms colonies per filter should be a reasonable count to ensure accuracy of the enumeration of coliforms [20], although the EPA guide for microbiological examination of water specifies the limit should be 80.

While membrane filter technique and multiple-tube fermentation technique are both considered as standard methods for coliform detection in water, the overall agreement of the two methods is about 94%, which suggests that they would generate very similar results if tested against the same water sample [14] [15].

4.2. Enzymatic Method

4.2.1. Defined Substrate Method: Presence/absence technique combined with multiple-tube fermentation technique

Enzymatic methods are preferred because of its ease of use, rapid detection rate and high sensitivity. The enzyme substrate test utilizes specific enzymatic activities of the coliform group, which was defined by multiple-tube fermentation technique using EC broth, to detect and enumerate coliforms. β-D-galactosidase, an enzyme produced by total coliform bacteria that catalyzes the breakdown of lactose into galactose and glucose, is used to detect and enumerate total coliforms; whereas, β-D-glucuronidase, an enzyme that catalyzes the hydrolysis of β-D-glucopyranosiduronic derivatives into their corresponding aglycons and D-glucuronic acid, and

whose activity was mostly limited to *E. coli,* has been used as an indicator for fecal coliforms [11].

A combined method of multi-tube fermentation technique and enzyme-based method are developed to overcome some limitations of the traditional methods. Water samples are added to a series of tubes containing chromogenic substrates, for example ortho-nitrophenyl- β-D-galactopyranoside (ONPG) or chlorophenonl red- β-D-galactopyranoside (CPRG), and are colorless immediately after sample addition. These tubes are then incubated at 35°C for 18 to 24 hours if ONPG is used, and for 24 hours when CPRG is applied. The β-D-galactosidase enzyme would hydrolyze the substrate and produce a color change to indicate a positive test [19]. Any yellow color presents in the tubes indicates a positive result for total coliform when ONPG is used as substrate; when CPRG is used, a red or magenta color would yield because of the hydrolysis reaction. When compared with multiple-tube fermentation technique, study shows that the defined substrate method generates about 2% false positive result, and the difference of the results from the two methods is not statistically significant [21].

In order to detect *E. coli* in water samples, a fluorogenic substrate, such as 4-methyl-umbelliferyl- β-D-glucuronide (MUG), is added to sense β-glucuronidase produced by *E. coli*. β-glucuronidase cleaves the fluorogenic substrate MUG through hydrolysis and releases the fluorescent product that can be observed when exposed under long-wavelength (365-nm) ultraviolet (UV) light. For both ONPG-MUG substrate and CPRG-MUG substrate, positive *E. coli* tests appear to emit blue fluorescence under long UV light. No additional confirmation tests need to be performed. The test has been compared with both multiple-tube fermentation technique and membrane filter technique, and has shown similar and effective results. Defined substrate method was able to recover 97% of the injured coliforms when compared with

multiple-tube fermentation technique [22], and showed similar sensitivity with the classical methods [23].

Colilert, a commercially available test developed based on enzymatic characteristic of coliforms, has been approved by AOAC. Colilert utilizes the defined-substrate technique that involves both chromogenic substrate ONPG and fluorogenic substrate 4-methyl-umbellifery-β-glucuronide (MUGlu). A yellow color indicates a positive test for total coliforms, and when the yellow test is exposed under long-wavelength UV light, a blue-white fluorescence implies a positive test for *E. coli*. An improved method of Colilert, Colilert-18 has also been approved by AOAC. Colilert-18, contains chromogenic substrate ONPG and fluorogenic substrate MUG, could provide result within 18 hours of incubation at 35°C, instead of 24 hours that is required by standard Colilert test.

The defined substrate method, as with other enzymatic tests, have a shorter time to result by omitting the fermentation procedure. Thus, instead of waiting at least 48 hours to get a result, Colilert 18 can provide a relatively accurate result within 18 hours. When Colilert 18 was test against membrane filter technique, it showed a false-positive rate below 2% [23]. Faster tests are desired for water quality surveillance to allow shorter response time if a hazard arises, but they are usually lack of high sensitivity, and therefore, could yield more false-negative results when compared with gold standard methods. Colilert 18, when compared with the membrane filtration method, for the detection E. coli was only 63.6% [24]. Among the false negative results, 29% was confirmed belonging to *Escherichia* genera, and 71% belonged to *Klebsiella* genera [23]. Other studies that were determined to evaluate the performance of defined substrate method have found similar result: Colilert has been shown to have a sensitivity of 58.5% when tested directly on pure strains of Enterobacteriaceae [25], although when tested directly on pure strains of E.

coli, a total of 95.5% of the cultures tested were β-D-glucurounidase-positive with 24 hours incubation, and 99.5% beta-D-glucurounidase-positive within 28 hours of incubation [26] [2].

4.2.2. Filtration Method

Filtration method is a combination of the membrane filter technique and enzyme-based method, where samples are filtered through 47-mm, 0.45-um pore size cellulose ester membranes that retain the bacteria present in the sample. The membranes are then placed into media for incubation. Similar to membrane filter technique, water sample size should be considered beforehand in order to get optimal test results. The same measure and suggestion for sample volume in membrane filtration method from APHA is applicable to filtration method as well, where an appropriate volume for drinking water should be 100 ml. The result of filtration test is expressed as counted colonies that can be converted to CFU/ml with calculation.

Both APHA and EPA have approved media m-Coli Blue 24 as a standard medium for filtration technique to test coliforms content in water sample. m-Coli Blue 24 medium could simultaneously detect total coliforms and *E. coli* within 24 hours. This specific medium contains antibiotics that inhibit the growth of background bacteria, as well as lactose, chromogen BCIG (5-bromo-4-chloro-3-indolyl- β-D-glucuronide) and a nonselective dye TTC (2,3,5-triphenoltetrazolium chloride). The dye TTC turns total coliform colonies red, while *E. coli* colonies are turned to blue to purple color and are distinguished from red colored other coliforms colonies because of the action of beta-glucuronidase on the chromogen BCIG. Coliform bacteria are therefore, defined as those that produce red colonies within 24 hours at 35°C on a medium containing lactose and a nonselective dye when m-Coli Blue 24 is used; and *E. coli* are defined as those microorganisms that produce blue/purple colonies [6]. Study shows very high sensitivity

and specificity when testing *E. coli* on drinking water samples; test showed only 2.5% false positive error and a zero percent false negative rate [28]. On total coliform test, however, both the false positive rate and false negative rate went up, where the false positive rate is 27% and false negative rate is about 2% [28]. The overall agreement for m-Coli Blue 24 medium against traditional membrane filter technique using m-Endo medium is 98.8% [28].

MI agar, another medium that has been approved by EPA and APHA, utilizes fluorogen 4-methylumbellifery-β-D-galactopyranoside (MUGal) and chromogen indoxyl-β-D-glucuronide (IBDG) to simultaneously detect β-galactosidase produced by total coliforms and β-glucuronidase produced by *E. coli* in water samples. A complete composition of MI agar includes the following compoents: proteose peptone No. 3, yeast extract, β-D-lactose, MUGal, IBDG, sodium chloride (NaCl), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium lauryl sulfate, sodium desoxycholate, agar, and reagent-grade distilled water. Coliform bacteria are defined as those bacteria that produce fluorescent colonies upon exposure to long wavelength ultraviolet (UV) light within 24 hour at 35°C on MI medium. The medium is able to generate results within 24 hours [6]. The procedure of conducting tests on MI agar is similar to on m-Coli Blue 24 medium; after bacteria in the sample has been retained on a 47-mm, 0.45-um pore size cellulose ester membrane filter, the filter is placed on MI agar for incubation at 35°C for up to 24 hours. The bacterial colonies that grow on the plate are inspected for the presence of blue color, which indicates the breakdown of IBDG by β-glucuronidase and therefore, implies the presence of *E. coli*. The plate would also be exposed to long wavelength ultraviolet light at 366 nm to check fluorescent bacterial colonies, which suggests the cleavage of MUGal by total coliform enzyme β-galactosidase [29]. No additional confirmation step is required for this agar. AOAC has also approved the MI medium

to be used as a standard test for coliform detection in water samples. On average, MI agar is able to recover 1.8 times as many total coliforms as m-Endo agar could, with significantly reduced background bacterial counts. The false-positive rate and false-negative rate for *E. coli* were both reported to be 4.3%. And the specificity for E. coli and total coliforms were 95.7% and 93.1%, respectively for MI agar [30]. MI agar has shown a sensitivity of 74.1% when tested directly on pure strains of Enterobacteriaceae [25].

Another supplemented agar-based medium, m-TEC agar, approved by EPA, can also be used to identify *E. coli* in water samples. The test does not involve any transfer of the membrane filter to another medium for incubation; only one medium is used throughout the whole detection process. Modified m-TEC agar contains protease peptone No. 3, yeast extract, lactose, sodium chloride, dipotassium phosphate, monopotassium phosphate, sodium lauryl sulfate, sodium desoxycholate, chromogen 5-bromo-6-chloro-3-indolyl-β-D-glucuronide, agar, and reagent-grade distilled water. The chromogen, 5-bromo-6chloro-3-indolyl-β-D-glucuronide, would be catabolized to glucuronic acid and produce a red or magenta-colored compound when the enzyme β-D-glucuronidase from *E. coli* is present. *E. coli* bacteria are defined as those which produce red or magenta colonies on the modified m-TEC agar. The procedure used in modified m-TEC agar is slightly different from other filtration methods. After a water sample is filtered through the membrane and bacteria are retained, the membrane filter is placed on the modified m-TEC agar to resuscitate the injured or stressed bacteria by incubation at 35 $\pm$ 0.5 °C for 2 hours. And then the filter is incubated at 44.5 $\pm$ 0.2 °C for another 22 hours. The target colonies, positive *E. coli* colonies, should appear red or magenta in color on modified m-TEC agar after the incubation period. The false-positive and false-negative rate are 13% and 4%, respectively [31][32].

The modified m-TEC agar test is mostly used for monitoring the quality of recreational water and marine waters. A verification procedure of the detected *E. coli* sometimes is required in order to get more information about the water source, or as a means of quality control of the test. For the verification test, the growth need to be transferred onto both a nutrient agar plate, and into a trypticase soy broth. Cultures are incubated for 24 hours at 35 $\pm$ 0.5°C in both cases. After incubation, a loopful of growth from the nutrient agar plate is transferred to react with cytochrome oxidase reagent. If deep purple color is observed within 15 seconds, the test is positive. In the meantime, some growth from the trypticase soy broth is transferred to an EC broth fermentation tube, a tryptone broth, and a Simmons citrate agar. Simmons citrate agar and tryptone broth are both incubated for 48 hours at 35 $\pm$ 0.5°C, and the EC broth is incubated at 44.5 $\pm$ 0.2°C in waterbath for 24 hours. After 48 hours incubation of tryptone broth, a trace amount of Kovacs indole reagent that contains ρ-dimethylaminobenzaldehyde, amyl or isoamyl alcohol, and concentrated hydrochloric acid, is added. A positive *E. coli* test should be: indole-positive and appear a deep red color in the tryptone broth culture, EC gas-positive, oxidase-negative, and does not utilize citrate, where the Simmons citrate agar remains its green color [32].

4.3. Molecular Method

Molecular methods are relatively new compared to traditional culture methods and enzymatic methods. They shorten the time to result by omitting the cultivation step and go straight for coliforms detection. Therefore, they are able to detect non-culturable bacteria within hours, which could be a good thing because those viable but non-culturable coliforms would not be underestimated. However, this ability of detecting non-culturable bacteria could also cause

overestimation of the target microbes because some of the non-culturable bacteria are actually dead and should not be included in test results. None of the molecular tests that were specifically designed for coliforms detection in water has been approved by the agencies this review covered, although AOAC did approve the BAX system PCR assay for *E. coli* O157:H7 detection in food.

4.3.1. Immunological methods: Enzyme-linked immunosorbent assay (ELISA) and Immunofluorescence assay or immune-enzyme assay (IFA)

Immunological methods utilize the specific recognition of antibodies for antigens and the presence of unique antigens on target cells. Depending on the targeted antigen of choice, the assays can detect antigens at specific family, genus, species, or serotype levels. Antibodies can be produced either monoclonal or polyclonal; whichever is more specific to the target organism. For example, an ELISA has been reported that uses a monoclonal antibody immunoglobulin G2a 898 against the enterobacterial common antigen (ECA), which is a lipopolysaccharide that is linked within the outer membrane of Enterobacteriaceae. The presence of enterobacteriaceae would be recognized through the use of this ELISA. Research has shown that the sensitivity of a direct test of ECA is not sensitive enough, but with pre-cultivation step of the water sample, so that the detection limit of ELISA would be reached (10^5 cells/ml), the test appears to be more sensitive with 98% sensitivity when compared with eyzam. A comparison test was conducted with multiple-tube fermentation technique detecting total coliforms to the ECA ELISA and 98% ECA ELISA positive samples were also positive using the multiple-tube fermentation. The false positive rate of ECA ELISA method was 0.8%, and false negative rate was about 1.2% [33]. The false positive result generated could be caused by *Pseudomonas*, *Aeromomas*, and *Bacillus* [33]. High non-targeted microflora levels could interfere the specificity of the ELISA method as well.

Immunofluorescence assay is another molecular method that utilizes the recognition of antibodies for antigens to detect specific microbes. Immunofluorescence assays can be performed either directly or indirectly; in a direct immunofluorescence assay, a specific antibody is directly conjugated with a fluorochrome, whereas in an indirectly immunofluorescence assay, two antibodies are used: the specific primary antibody that is binded with the targeted antigen and a fluorochrome-labeled antibody that is conjugated onto the primary antibody. Enumeration of fluorescently labeled cells can be detected by epifluorescence microscopy or solid-phase cytometry after filtration of the water sample, or by simply by flow cytometry. When compared with traditional method membrane filtration technique, immunofluorescence assay exhibited higher efficiency of detecting fecal coliforms that measured by CFU/ml, and showed 2 orders of magnitude higher than membrane filter technique [34]. However, when large amount of non-targeted bacteria is present, the immunofluorescence assay appears to be less effective. A threshold has been estimated: the targeted bacterium has to be at least 1% of the total population in order to perform the assay [34].

One major limitation of immunological methods in general when applied to drinking water quality monitoring is that the low number of targeted cells in the samples could result in false negative results. Methods developed more recently have been trying to diminish this disadvantage by concentrating the targeted cells in advance. Some studies tried to apply the immunomagnetic separation method (IMS), where magnetic beads are coated with monoclonal or polyclonal antibodies to concentrate the targeted cell in order to minimize the background interference. IMS has been mainly used in food samples, not much research on water samples have been conducted [11].

4.3.2. Nucleic acid-based methods: Polymerase Chain Reaction Methods

Nucleic acid methods exploit molecular hybridization properties that involve the complementary sequence recognition between a nucleic probe and a nucleic target. Tests utilizing this specific recognition are usually highly specific and sensitive. PCR enables the amplification of the target DNA fragment and thus, increase the concentration of target fragment and heighten the chance of detecting water samples with low coliforms concentration. The replication consists of a chain reaction catalyzed by Taq polymerase (a DNA polymerase) and the use of oligonucleotidic primers to initiate the replication cycles. The specificity of detection depends on the choice of primers, whether they complement the target sequence efficient enough, and on the hybridization temperature. The most common PCR method applied to detect and identify bacteria is an in vitro cycling replication after a DNA extraction, where cells are lysed and filtered through a membrane to get its DNA extract. A full PCR amplification process is completed in three steps: denaturation, annealing, and extending. The denaturation step would denature the double stranded DNA to single stranded. And then primers are annealed to the single-stranded DNA at a specific hybridization temperature, which usually is determined by the length and the composition of the primer, and is about 5°C lower than the melting temperature of the primer chosen [35]. Starting with the primers, new strands of DNA are extended by the enzyme, DNA Taq polymerase. The DNA target is amplified 20 to 40 cycles and detected after electrophoresis on agarose gel and staining by an intercalating dye.

Primer selection is crucial for insuring the specificity of the assay. Primers designed based on lacZ gene have been used for total coliforms detection. lacZ gene encodes for the expression of β-galactosidase [36], and a PCR-based assay was able to detect over 90% of the coliform strains defined by traditional culture-based method [25], where the 10% miss is mostly

contributed by Serratia [25]. The sensitivity of lacZ PCR is calculated to be 93% with 70% specificity when compared with membrane filter technique, and the overall agreement of the two tests is 84% [36]. And when compared with Colilert test with substrate ONPG, the overall agreement of the test is 92% with 95% sensitivity and 85% specificity [26] [36]. In order to increase the specificity of PCR test, some studies proposed the use of multiplex PCR, which consists of simultaneously amplifying several different DNA fragments by blending more primers in to the assay. However, it did not perform well due to the composition and the length of the primer oligonucleotide. Moreover, the relatively large size of the amplified DNA fragments may also influenced negatively on the result [11].

Another frequently used target gene sequence to detect enterobacteriaceae, and therefore, to detect coliforms, is the 16S rRNA. 16S rRNA sequence has been used very often when identifying bacteria that are categorized based on phylogenies, and the results are usually specific. When used to detect total coliforms, 16S rRNA test also shows a very high sensitivity of 98% and specificity of 92% [25].

For specific detection of *E. coli*, primers developed based on uidA gene were proposed; uidA gene encodes the β-D-glucuronidase enzyme [37]. The sensitivity of using PCR method detecting *E. coli* was reported to be relatively high when compared against MUG-based defined substrate tests. When compared with EC-MUG test, standard *E. coli* test described in defined substrate method, the overall agreement of uidA PCR test is 83%, with 93% sensitivity and 76% specificity [36].

Molecular methods in general cannot reflect the physiological state of the target microorganism. Even with sufficiently treated water samples, as long as the DNA fragments of coliforms or the antigens still exist in the sample, test would appear to be positive. Some has

proposed to incorporate immunofluorescence test with 16S rRNA test. Three antibodies, anti-DnaK, anti-Dps, and anti-Fis, are designed to conjugate with the three stage-specific growth proteins of coliforms respectively. DnaK is a metabolically stable protein that is nearly invariant with growth state, and can be used as permeabilization controls. Dps homologs are abundant in stationary-phase or dormant cells, but are virtually undetectable in growing cells; and therefore, are inversely correlated with growth rate. In contrast, Fis homologs are abundant in growing cells and nearly undetectable in stationary-phase cells. The physiological state of total coliforms in water samples are then estimated by the measurement of DnaK, Dps, and Fis abundance by protein profiling [38]. However, no data result was provided by the test could validate the method and prove it performed better than standard 16S rRNA test.

Another approach to eliminate the effect of the dead cells on PCR test result is to add propidium monoazide (PMA) to the sample before running the test. PMA, has been proved to be able to distinguish dead cells from the live ones and extraneous DNA, can permeate only the membrane-compromised cells and bind to extraneous dsDNA in the water samples that has been causing the problem. The photoinducible azide group of PMA allows the chemical to covalently intercalate the DNA of dead cells upon exposure to bright light and result to be undetectable during the subsequent genomic DNA extraction process [39]. Some real-time PCR assays have adopted the use of PMA and the research is still ongoing [40].

The table in the following page serves as a summary of the performance of some techniques that have been mentioned in this review. It demonstrates the abilities of these tests to detect total coliforms, where the detected microorganisms under each test are marked with "+" [3] [29] [43] [44]. For multiple-tube fermentation technique, however, only the microorganisms that the technique failed to detect is marked out as "Neg" standing for negative results [45].

	Test Methods					
Microorganisms	**Multi-tube Fermentation**	**MI agar (fluorescent)**	**Colilert (yellow)**	**DC agar with BCIG (pink)**	**lacZ PCR**	**16S rRNA PCR**
Budvicia aquatica						+
Buttiauxella agretis		+	+		+	+
Cedeca davisae					+	+
Cedeca lapagei		+			+	+
Cedeca neteri		+			+	+
Citrobacter amalonaticus	Neg	+			+	+
Citrobacter braakii		+	+		+	+
Citrobacter farmeri		+			+	+
Citrobacter freundii		+	+		+	+
Citrobacter gillenii		+	+		+	+
Citrobacter koseri		+	+		+	+
Citrobacter murliniae		+	+		+	+
Citrobacter sedlakii		+	+		+	+
Citrobacter werkmanii		+	+		+	+
Citrobacter youngae		+	+		+	+
Cronobacter muytjensii		+	+			
Cronobacter sakazakii			+		+	+
Enterobacter aerogenes	Neg	+	+		+	+
Enterobacter amnigenus		+			+	+
Enterobacter asburiae		+	+		+	+
Enterobacter cancerogenus		+	+			
Enterobacter cloacae subsp. Cloacae		+	+		+	+
Enterobacter cloacae subsp. Dissolvens		+	+		+	+
Enterobacter enterogenes		+	+		+	+
Enterobacter gergoviae		+			+	+
Enterobacter hormaechei		+	+		+	+
Enterobacter intermedius		+			+	+
Enterobacter pyrinus		+			+	+
Escherichia balttae					+	+
Escherichia coli	Neg	+	+		+	+
Escherichia fergusonii		+			+	+
Escherichia hermanii					+	+
Escherichia vulneris		+	+		+	+
Ewingella americana						+
Hafnia alvei		+			+	+
Klebsiella oxytoca	Neg	+	+	+		+
Klebsiella pneumoniae		+	+	+	+	+
Kluyvera ascorbata		+	+	+	+	+
Kluyvera cryocrescens		+	+	+	+	+
Kluyvera georgiana		+	+	+	+	+
Kluyvera intermedia		+		+		
Leclercia adecarboxylata		+	+	+	+	+
Moellerella wisconsensis		+	+	+	+	+
Pantoea agglomerans					+	+
Pantoea dispersa					+	+
Rahnella aquatilis		+	+	+	+	+
Raoutella omithinolytica		+	+		+	+
Raoutella planticola		+	+	+	+	+
Raoutella terrigena		+			+	+
Salmonella bongori		+	+		+	+
Salmonella enterica subsp.enterica			+			+
Serratia entomophila		+			+	+
Serratia ficaria		+				+
Serratia fonticola		+		+		+
Serratia grimesii		+	+		+	+
Serratia liquefaciens		+	+		+	+
Serratia marcescens		+	+			+
Serratia odorifera		+	+		+	+
Serratia plymuthica		+	+			+
Serratia proteamaculans subsp.quinovora		+	+			
Serratia rubidaea		+	+	+	+	+
Shigella spp.					+	+
Trabulsiella guamensis		+	+		+	+
Yersinia aldovae					+	+
Yersinia bercovieri		+			+	+
Yersinia enterocolitica						+
Yersinia frederiksenii		+	+		+	+
Yersinia intermedia		+			+	+
Yersinia kristensenii					+	+
Yersinia mollaretii					+	+
Yersinia pseudotuberculosis					+	+
Yersinia rohdei		+			+	+
Yersinia ruckeri					+	+
Yokenella regenburgei		+			+	+

5. Discussions and Conclusion

This purpose of this review is to give a comprehensive understanding of the methods that have been used for coliform detection, which include both total coliforms detection and fecal coliforms confirmation. Three categories of methods have been included in this paper, the traditional culture-based methods, enzymatic methods, and molecular methods. Under each category, some approved methods are listed and described, where the performance of each technique has been compared through sensitivity and specificity results.

All of the methods mentioned in this review have individual limitations. The coliform group, unlike other bacteria groups that are categorized based on bacteriology, refers to many bacteria that do not share many characteristics in common and are different in definition in biochemistry and in serology. Initially, coliforms are defined based on its lactose fermenting characteristics, where the multiple-tube fermentation and membrane filter technique are developed to test this specific feature of coliforms. However, some members in the coliform groups only exhibit limited ability to ferment lactose [41]; some can only produce gas from lactose very slowly or in negligible amount at 37°C or 20°C, where the fermentation temperature is different from the typical lactose fermenting temperature shared by the majority in the coliform group. Although only few members in the coliform group have shown this aberrant traits, those members are responsible for generating false negative test results and leading to underestimation of the coliforms content in the water sample. Moreover, the lactose fermenting characteristic is not limited to the coliform group; *Aeromonas* and *Psedomonas* genera can also ferment lactose, and they have proved to be one of the major interfering microorganisms and are responsible for the false positive results in traditional techniques.

A major reason that many new techniques are developed to replace the traditional ones is that the traditional ones take too long to yield results. The multiple-tube fermentation technique takes about 96 hours (48 hours incubation time plus 48 hours confirmation test) and the membrane filter technique requires at least 24 hours incubation time before results can be produced for both total coliform and fecal coliform tests. Moreover, often times, verification of the result from membrane filter technique is recommended, which requires at least another 4 hours. Later developed enzymatic methods and molecular methods are determined to decrease the turn around time as well as to detect those aberrant coliforms that only possess limited ability to ferment lactose together with the typical ones in a single test. Enzymatic methods exploit the activity of β-D-galactosidase and β-D-glucuronidase, whose activities are limited only to the coliform group and E. coli, respectively. The defined substrate technique requires a minimum 18 hours turn around time to result for total coliforms and for *E. coli* simultaneously, and no confirmation step is needed. Filtration methods can also give results for both total coliforms and *E. coli* within 24 hours. Molecular methods can even shorten the turn around time to a few hours since they do not require any cultivation step, and neither do they require confirmation tests.

Another problem with the membrane filter technique is its incapability of recovering stressed or injured coliforms. Those stressed coliforms caused by disinfection could neither ferment lactose efficiently nor form colonies on the medium. Hence, they would not be detected by the test, where the abundance of coliforms would be underestimated. Improved medium such as m-T7 medium has been proposed to solve the problem, however, the medium is reported not being effective enough when recovering coliforms that are stressed other than by chlorine; research showed no significant difference was observed between the m-T7 medium and m-Endo medium [18]. Adding catalase, sodium pyruvate or both in the medium is another approach that

has been proposed to address the same problem. The addition is used to compensate the lost catalase content to reduce the increased level of hydrogen peroxide in coliforms due to disinfection. Some promising outcomes have been yield with this approach, but it is not widely accepted nor used.

Neither enzymatic tests nor molecular methods share the same concern since neither of them is detecting the coliform group by exploiting its lactose fermenting characteristics. Moreover, the molecular techniques do not require any recovery phase to detect coliforms. For enzymatic tests, even though a recovery stage is applied, no such problem has been reported. An explanation for this could be that for the defined substrate technique, the method used for recovering stressed or injured coliforms is adopted from multiple-tube fermentation technique, where no evidence shown that the technique is not efficient enough to recover stressed cells. And for filtration technique, even though the recovery phase utilize the same mechanism as membrane filter technique, with different medium and different testing target of the test, it is reasonable that the β-D-galactosidase activity of those stressed and injured coliforms is detectable and would generate a positive test for enzymatic test, but the injured bacteria are not recovered well enough to give a positive lactose fermenting result.

For both traditional and molecular techniques, the sensitivity and specificity are influenced by the amount of non-coliform microorganisms in the background. In multiple-tube fermentation technique, large amount of background microorganisms significantly increase the false positive rate and false negative rate of the presumptive test. The distinction between a positive test and a negative test for multiple-tube fermentation test is rather objective, where the result is judged by formation of gas regardless of the amount generated during a certain period of time. In a turbid sample, such phenomenon could easily be misinterpreted in either direction.

Moreover, background bacteria such as *Aeromonas* and *Psedomonas* that could ferment lactose could also be responsible for the confusion.

In membrane filter technique, crowed background bacteria could interfere the recovery rate of coliforms and reduce coliform colonies on m-Endo media. It is possible that the nutrients that suppose to recover coliforms are preempted by those background bacteria, and not enough are left for the intended microorganisms. So, not all the culturable coliforms are able to form colonies on the medium, and the plates may be crowded with non-coliform colonies. Defined substrate technique is designed to eliminate the background interference; the media used in the method has added antibiotics or other inhibitory chemicals to prevent background bacteria from growing, so that the media would only culture the target microbes to ensure proper recovery of the coliforms.

The decrease in sensitivity of enzymatic method has been considered as a major trade off for the rapid detection. In the case of using Colilert 18, where defined substrate technique is applied, , has significantly reduced the turn around time. However, the shorter the turn around time, the less time the target microbes get to recover, and the higher chance that more false negative results are generated. When compared with filtration technique that utilizes the modified m-TEC agar to detect *E. coli*, the sensitivity of Colilert 18 was only 64%. Even on pure strains of *E. coli*, defined substrate technique cannot achieve 100% positive result after 28 hours of incubation, although this may also be related to the physiological state of the bacteria: some of the bacteria in the test are simply not culturable.

Molecular techniques are the most recently exploited approach to detect coliforms in water quality surveillance. They allow more rapid detection than enzymatic approaches by bypass all the incubation steps that have been causing inaccuracy in traditional methods and

enzymatic methods. But they are not perfect either. An ultimate problem with immunological approach is to find the common antigen of the coliform group. The most frequently used target antigen is the enterobacterial common antigen (ECA), which is a family-specific surface antigen that is shared by all members of the Enterobacteriaceae family and also is restricted to this family [42]. And immunological assays using ECA could only detect coliforms by directly detecting enterobacteriaceae first. Although many members of the coliform group belong to enterobacteriaceae family, there are still members coming from other bacteria families. Meanwhile, many members of the enterobacteriaceae family are not coliforms. The use of non-specific antigen is one of the reasons of why the immunological approach usually is not sensitive enough. However, to our current knowledge, no common antigen is shared by all coliform bacteria, which makes the immunological approach harder to reach its optimal potential. PCR approach shares the similar default difficulty when identifying the target gene. 16S rRNA gene, which is originally used to detect enterobacteriaceae, is used to detect coliforms. Despite high sensitivity and septicity are resulted from the test, it lacks solid reasoning that supports the use of this gene in coliform detection. Other commonly targeted genes used in the PCR techniques are the lacZ gene and uidA gene that are associated with the expression of β-galactosidase and β-glucuronidase, respectively. And this PCR test is established based upon the enzymatic definition of coliforms.

Molecular techniques cannot tell the physiological state of the target, which often cause an overestimation of the actual coliforms counts. Some authors have suggested a combined technique that utilizes immunefluorescence and stage-specific growth protein to determine the viability of the bacteria; others suggest addition of chemicals such as PMA to eliminate the

influence of the extraneous DNA. Although neither of the approaches has been validated, they seem to be some prospective techniques to be used in the future.

The paper includes only some of the techniques that are approved by related agencies and have been most used. It is a brief overview of coliform detection methods that mainly focus on drinking water. Some understanding and analysis on a few limitations of each method are discussed in order to give some opinions that could be considered in future studies about coliforms. The techniques covered in this review are exploiting different characteristics of coliforms, and thereby identify coliforms. The definitions of coliforms vary with each individual detecting methods. The paper is served as a tool to appreciate some of the current methods used in coliform detection on water samples and hopefully to point some directions for future researches on the subject matter.

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