MICROBIAL ANALYSIS AS INDICATORS OF POLLUTION IN WASTEWATER OF HLAINGTHARYA INDUSTRIAL ZONE

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Abstract

The present work was conducted to study the status of some indigenous bacterial species in wastewater from factories of noodles, confectioneries and rice vermicelli in Hlaingtharya industrial zone near Yangon city of Myanmar. The study was done from June to August, 2017 for preliminary studied. All samples were showed *Escherichia coli* positive on 3M petriflim. Among them, four *E.coli* isolates were tested for their antibiotics susceptibility patterns with Ampicillin and their generation time were studied. All *E.coli* isolates showed ampicillin resistance and generation time was between 20 to 22 min. Subsequently, genomic DNA and plasmid DNA profiling those antibiotics resistant *Escherichia coli* strains was done for future reference in further works on pollution estimation in this industrial zone.

Keywords: *Escherichia coli*, petriflim, Genomic DNA, Plasmid DNA

Introduction

Indicator organisms are bacteria that are used as a sign of quality or hygienic status in a food, water, or environment. The definition of the word "indicator," . . . in fact, includes the concept of the indicator organism, i.e., something "so strictly associated with particular … conditions that its presence is indicative of the existence of these conditions"(Merriam-Webster Online, http://www.m-w.com/cgi-bin/dictionary, 2018).

There is an extensive literature which stresses deterioration of water quality. The addition of various kinds of pollutants and nutrients through the agency of sewage, industrial effluents, agricultural runoff etc: into the nature natural water bodies brings about a series of changes in the physiochemical and other characteristics of water, which have been the subject of several investigations (Tiwari and Mishra, 1986 and Khulab, 1989).

The major microbial pathogens in water are bacteria, viruses, fungi and protozoan parasites. Bacteria pathogens are mostly present in feces and a wide variety can be present in wastewater due to fecal contamination. The discharge of untreated or inadequately treated wastewater into the environment can have negative impact on human health due to the release of pathogenic microorganisms into water which could lead to serious health diseases (Rosario *et al*., 2009). Water that is contaminated with microbial pathogens is a medium for several waterborne diseases, such as cholera, typhoid fever, shigellosis, salmonellosis, campylobacteriosis, giardiasis, cryptosporidiosis and Hepatitis A (WHO, 2004). Several pathogenic organisms in contaminated water are the basic causes of gastrointestinal illnesses in human. Some of the pathogens are known to cause several outbreaks of diseases by releasing toxins in the human body (Krauss and Griebler, 2011).

Indicator organisms were first used in the testing of water supplies for sanitary quality. The mid to late 1800s were marked by huge developments in the sciences of public health and microbiology. Indicator organisms in wastewater are organisms whose presence suggests the

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presence of a pathogen in wastewater. The density of an indicator organism is always associated with health hazards and several sources of pollution. It is indicated that for an organism to qualify as an indicator organism of a particular pathogen, it must be continuously and totally related to the source of the pathogen and be abundant enough to provide appropriate and exact mass concentration of the level of pathogen in relation to high risk of illness. Also, an indicator organism should have resistant ability to disinfectants, environmental stress and toxic materials that may be present at the source of the pathogen (Berg, 1978; Galveston Bay Centre, 2002).

Many published standardized methods exist for the detection and enumeration of Enterobacteriaceae, coliforms and *E. coli* in foods including international standard methods like those published by the International Organization for Standardization (ISO). Nowadays, coliforms bacteria isolated from foods that indicate poor hygiene or inadequate processing, process failure and post-process contamination of foods. *E. coli* is commonly used to provide evidence of faecal contamination in certain foods and is used as an index organism for the presence of enteric pathogens such as *Salmonella.* (Muytjens *et al.,* 1988).

The use of *E. coli* as an indicator organism is somewhat restricted by the fact that *E. coli* is not a single species; certain genera of the coliform group such as Proteus and Aerobacter are normally found outside the human intestinal tract in soil; other organisms found in water that do not represent fecal pollution possess some of the characteristics attributed to *E. coli* and *E. coli* identical to that found in humans is also found in the intestinal tract of other warm-blooded animals. However, primarily, studies have shown that *E. coli* is a much better indicator of disease risk than is faecal coliform, EPA has therefore, recommended that *E. coli* be used as a criteria for classifying waters for fresh water contact recreation. (EPA, 1986 and Hoffmann, Sturenburg and Heesemann, 2013) .

This study was aimed at analysis of microbial pathogen indicators as microbial pollutants for contaminated wastewater from three food industries in Hlaingtharya Industrial zone and focus on determination of *E.coli* strain and their antibiotic resistance, growth rate and genomic DNA and plasmid DNA profile of this species. This finding will provide references for future work on pollution estimation especially for the *E.coli* bacteria detection at the molecular level.

Materials and Methods

Sample collection and identification of *E. coli*

The wastewater samples were collected and analysed from the disposal of three factories sites including noodles, confectioneries and rice vermicelli factories in Hlaingtharya industrial zone near Yangon city of Myanmar. This study was done during June to August, 2017 as preliminary research.

The wastewater samples (one liter each) were collected in triplicates per sites. Samples were collected during the day at 9.00 am from each sampling site and collected aseptically in sterile containers and placed in a cooler box and transported to the Microbiology Laboratory of Zoology Department, West Yangon University within two hours after collection. One mL of each diluted samples cultivated and identified on 3M Petriflim^{TM} *E.coli* count plate (Thermofisher Scientific, Austrialia) according to their instruction and enumeration of *E.coli* was done with Plate Reader (TICO, USA).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084), according to the Clinical and Laboratory Standards Institute (CLSI, 2009) guidelines. The antimicrobial agent tested and their corresponding concentrations was Ampicillin (25 μg/disk), after incubating the inoculated plates aerobically at 37 °C for 18 to 24 h, the susceptibility of the *E. coli* isolates was measured and the results were recorded in accordance with criteria provided by CLSI. *E. coli* ATCC 25922 was used as quality control organisms in antimicrobial susceptibility determination.

Determination of generation times

Four isolates of ampicillin resistance *E.coli* strains tested for their generation time. Each *E.coli* samples were diluted into 10^1 to 10^{10}, and incubated on nutrient agar and incubated at 37 ^{0}C checked for their growth at every one hour and recoded as bacterial colony/mL. 10^5 dilution factors was used for determination of their generation time and calculation method was followed by Stanley *et al.,* 1994.

Extraction of Genomic DNA

To prepare genomic DNA from cultured *E.coli* bacterial, the bacteria from 3 ml of liquid media were used for extraction of genomic DNA used with Pure Link Genomic DNA Mini Kit (Invitrogen, Life Technologies, 50 preps, USA) according to the manufacturer's instructions (Invitrogen Test kit, Germany). Centrifuged at 16000 Ug for 30 min, and the pellet was used for DNA preparation with the kit mentioned above.

Extraction of Plasmid DNA

To prepare plasmid DNA from *E.coli* bacterial strains, the bacteria from 3 ml of liquid media were used for plasmid DNA isolation according to the manufacturer's instructions (QUIAGEN Plasmid Mini Kit, Germany).

Qualitative and Quantitative analysis

Extracted genomic DNA and plasmids DNA were separated by horizontal agarose gel electrophoresis (100 DVC, 1% agarose gel, 1X TAE bufferin 30 mins). After Etbr staining, DNA was visualised and digitalized images captured under UV light trans illumination (Gel Imager, Thermofisher scientific, Co. Ltd) the molecular weight of each plasmid, Gene Ruler™ 1kb DNA Ladder marker was used to estimate the molecular mass. The Nanodrop (ND 1000, Thermofisher scientific, Germany) was employed to measure optical density at 260 nm and 280 nm for quantity of DNA concentration.

Results and Discussion

Escherichia coli is the best indicator of fecal contamination from human and animal wastes. *E.coli* presence is more representative of fecal pollution because it is present in higher numbers in fecal material. Baudizsova (1997) found that the other thermotolerant and total coliforms were capable of growth in non-polluted river water while *E.coli* was not, and supports a recommendation for *E.coli* to use as the sole indictor bacteria for recent fecal contamination (Tallon, *et.al.,* 2005).

According to this reason, the present research work was conducted to isolate *E. coli* from three food processing factories including noodles, confectioneries and rice vermicelli factory in Hlaingtharya industrial zone near Yangon city as a preliminary research during June to August, 2017. In this study, after incubation on 3M Petriflim^{TM} *E.coli* count plate showed all samples are *E.coli* positive, produce blue to red blue colonies, and give lactose fermentation produce gas. It is due to plate contain Violet Red Bile (VRB) and indicator of glucuronoidase activity.

Table 1 Results of *E.coli* isolates from three different food processing wastewater effluent drainage.

Factory	**Total percentage**
Noodle processing factory	100%
N 1	+
N 2	+
N 3	+
Confectionary processing factory	100%
C 1	+
C 2	+
C 3	+
Vermicelli processing factory	100%
V 1	+
V 2	+
V 3	+

Table 2 Growth as colony count/mL of *E.coli* isolates from three food processing factories. (incubation at 37 ^{0}C)

Isolate Number	**Sampling intervals (hrs)**								
	0:00	**1:00**	**2:00**	**3:00**	**4:00**	**5:00**	**6:00**	**7:00**	**8:00**
1	0	72	102	180*	102	90	82	0	0
2	0	40	100	191*	95	63	49	0	0
3	0	38	61	148*	34	10	30	40	0
4	0	38	60	150*	52	50	49	42	0

(Nutrient agar = 20 mL agar plate + 1 mL sample; Dilution factor 10^{4} ; * = Peak growth)

In noodle factory, mainly uses with egg, flavor, instant pigments, yeast, starch and large volume of water are using for cleaning. In confectionary factory, mainly use with sugar, water, milk, flavoring materials, nuts, vegetable oils, scereals, eggs, instant pigment, baker yeast and liquid sugar. All of these wastes contain mainly nutrient for bacteria. Vermicelli factory use with dry rice flour and yeast are high content with starch and heavy particulates, this will term to more favorable for bacteria growth in their wastewater effluent. All factories produce large amount of waste characterized by high concentration of organic materials and nutrients. Their effluent discharges directly to the environment and there is no proper treatment process. These wastewater are making bad odor in environment.

E. coli bacterial species has a great capacity to accumulate resistance genes, mostly through horizontal gene transfer. In this study, four *E.coli* isolates from three different factories were tested with ampicillin antibiotic. All are showed resistance on this antibiotic. Ampicillin

resistance strains are important in clinical microbiology. They are not only for its effects on human health but also for potential source of transferring the antibiotic-resistant genes to other important pathogenic serotypes through horizontal gene transfer between bacteria and that contributing to the increase of the resistant genes in the environment (Sarina Pignato, *et al.*, 2010).

Table 3 Generation time of *E.coli* isolates from three factories

Tested samples	Initial number of bacteria colony x 10^5/mL	Final number of bacteria colony x 10^5/mL	Generation Time (min)
1	10.20 x 10^5	18.00x 10^5	20.42
2	10.00 x 10^5	19.10x 10^5	22.36
3	6.10 x 10^5	14.80x 10^5	20.52
4	6.00 x 10^5	15.00x 10^5	21.36

Generation times are varies among in bacteria, is controlled by the nature of bacteria species, *E.coli* has every 20 to 30 minutes for generation time (Stanely *et al*., 1994). Four isolates are tested their generation time and all *E.coli* isolates showed between 20.42 to 22.36 minutes of their generation time. These results are reveled with similar to other studies. According to this study, little variation are occurred may be due to the medium that provides energy source and more of biosynthetic intermediate that the cell would otherwise that make to itself.

Clinical isolates of *E.coli* are revealed a greater degree of genomic size variation than the detected among the natural strains that occur in wastewater (Bremnner, *et al.*, 1972). That varies estimate ranging from 3.8 to 4.8 Mb. In this study, all *E.coli* isolates were greater than the 20 to 50 kb (Genomic DNA) and less than the 30 kb (Plasmid DNA). It was dependent on the GC content of the genome as well as the amount of unique sequences; it is difficult to establish reflect actual variation in genome size. The isolation of plasmid DNA from bacteria is a crucial technique in molecular biology and is an essential step in many procedures such as cloning, DNA sequencing, transfection, and gene therapy. These manipulations require the isolation of high purity plasmid DNA. The purified plasmid DNA can be used for immediate use in all molecular biology procedures such as digestion with restriction enzymes, cloning, PCR, transfection, in vitro translation, blotting and sequencing. The purified plasmid DNA can be used for immediate use in all molecular biology procedures such as digestion with restriction enzymes, cloning, PCR, transfection, in vitro translation, blotting and sequencing (Anonymous, 2018).

Table 4 Genomic DNA, Plasmid DNA concentrations and Purity of DNA from Isolated *E.coli* strains (A 260/280)

Tested samples	Genomic DNA (ng/µL)	DNA purity	Plasmid DNA (ng/µL)	DNA purity
1	38.50	1.90	19.60	1.85
2	31.90	1.96	19.10	1.86
3	26.50	1.83	20.20	1.84
4	28.00	1.80	18.40	2.00

To the degree that naturally occurring microbial pathogens become a significant public health concern, completely new test procedures may have to be developed. Furthermore, while *E.*

coli is specific for faecal contamination, there are three inherent problems of using *E. coli* as a confirmation of faecal contamination: (i) it is outnumbered by other types of fecal bacteria making it more difficult to find; (ii) it does not survive for long outside of the gut; (iii) it can be found in pristine environments in the tropics. Therefore, the absence or presence of *E. coli* via a culture test does not absolutely confirm the absence or presence of faecal contamination. The *E. coli* tests used today as an indication of fecal contamination are commonly culture tests although that need to study with the sensitivity test, serotype, molecular test for the pathogen *E.coli* strain (USEPA, 2000). Thus in recent study *E.coli* isolates were used as indicator and further study with the antibiotic resistance test and plasmid profile for the further study of PCR detection for pathogenic strain.

Many studies of antibiotic-resistant bacteria in the aquatic environment but little work has been done to assess the prevalence of drug-resistant bacteria in water and their relationships to antibiotic-resistant microorganisms in untreated source waters. They have found increased rates of resistant bacteria in drinking water within the distribution net by standard plate-count experiments, and have concluded that the treatment of raw water and its subsequent distribution select for antibiotic-resistant bacteria (Armstrong, Shigeno, Calomiris, and Seidler, 1981).

In agreement with these data, increased phenotypic resistance rates were also detected in *E.coli* isolates. Additionally, plasmid profile investigations concerning the underlying resistance mechanisms were performed for further study of antibiotic resistance gene. The occurrence of the ampicillin resistance genes of *E.coli* bacteria confirmed for the influence of the water sources on the study area.

Plate 1. Antibiotic susceptibility patterns of *E.coli* isolates on Muller Hinton agar tested with ampicillin
1-4 = sample number of *E.coli* isolates; C = control sample

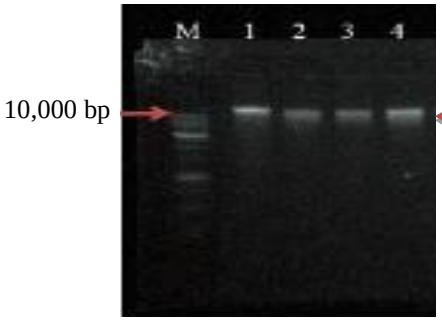

Plate 2 Gel electrophoresis image of extracted genomic DNA from *E.coli* isolates of three sample sites (Lane M = 1Kb plus maker ; Lane 1-4 = *E.coli* isolates)

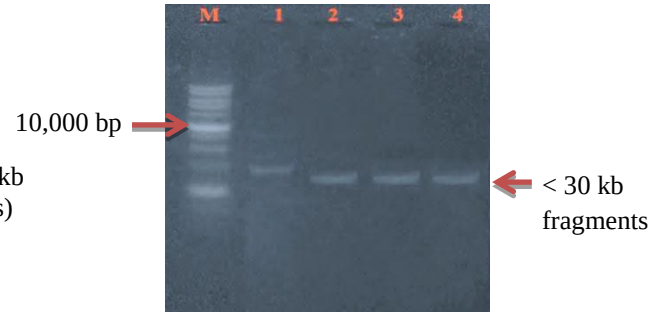

Plate 3 Gel electrophoresis image showing plasmid profiles extracted from *E.coli* isolates of three sample sites (Lane M = 1Kb plus maker ; Lane 1-4 = *E.coli* isolates)

Conclusion

In conclusion it's clear that *E. coli* appears to be the best indicator of bacteriological quality of water, primarily because of the, availability of affordable, fast, sensitive, specific and easier to perform detection methods for *E. coli*. However the fact remains that the life span of *E. coli* in water is short, thus it best determines, recent contaminations. It is therefore important that there is continuous monitoring for *E. coli* to determine the bacteriological quality of water. Indicator organisms continue to serve important functions in microbiological testing programs. Further research is required in order to evaluate the health risks of using reclaimed water harboring antibiotic-resistant bacteria for drinking, agricultural, and recreational purposes**.**

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