ANTIBIOGRAM, SCREENING OF BIOFILMS, PLASMID PROFILING AND OCCURENCE OF SHIGA TOXIN GENES OF *Escherichia coli* ISOLATED FROM RAW SALAD VEGETABLES

A Dissertation
Submitted to the **Department of Microbiology**,
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Nepal, in Partial Fulfillment of the Requirements for the Award of
Degree of Master of Science in Microbiology
(Public Health Microbiology)

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RECOMMENDATION

This is to certify that **Ms. Sujata Rai** has completed this dissertation work entitled **"Antibiogram, Screening of Biofilms, Plasmid Profiling and Occurrence of Shiga Toxin genes of *Escherichia coli* Isolated from Raw Salad Vegetables"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (Public Health) under my supervision. To my knowledge this work has not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Prince Subba** this dissertation work of **Ms. Sujata Rai** entitled **"Antibiogram, Screening of Biofilms, Plasmid Profiling and Occurrence of Shiga Toxin genes of *Escherichia coli* Isolated from Raw Salad Vegetables"** has been approved for the examination and is submitted to the Tribhuvan University in Partial fulfillment of the requirements for M.Sc. degree in Microbiology (Public Health).

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ABSTRACT

Raw salad vegetables are an essential ingredient of a healthy diet. Minimally processed food or other raw vegetables have become popular since it suits the present necessity as there is no need of further preparations. Although, they pose great health benefits, the consumption of fresh vegetables has also been associated with risk for consumers. Presence of multidrug resistant bacteria in salad vegetables has emerged as a potential health risk to the consumers worldwide. *E. coli* is one of the most common foodborne pathogen and also considered to be an important reservoir of transferable antibiotic resistance. The present study dealt with the isolation of *E. coli* from raw salad vegetables and their antibiogram was determined. The present study revealed the prevalence of *E. coli* isolates was at 47.27% with prevalence of MDR isolates at its highest peak (100%). Plasmid profile showed that all the *E. coli* isolates harbored plasmid mostly of size of >10kb. Two isolates possessed double plasmids of sizes of approximately 3kb and >10kb while one isolate showed triple plasmid bands of sizes of approximately 2.7kb, 3kb and >10kb. Screening of biofilm formation was done by CRA and TM. MIC and MBC values of pesticides (chlorpyrifos and cypermethrin) by broth microdilution method using 96-well microtiter plate showed that biofilm producing *E. coli* isolates were more resistant to those pesticides as compared to biofilm non-producers. On performing the PCR assay for the detection of Shiga-Toxin genes, four of *E. coli* isolates exhibited stx_1 gene and none of the isolates exhibited stx_2 gene. The raw salad vegetables sold in vegetable markets of Dharan were highly contaminated with *E. coli*. The prevalence of MDR *E. coli* implies the emergence of MDR bacteria in salad vegetables at huge extent. The presence of STEC indicates the possible foodborne illnesses to the consumers. Also, biofilm producing *E. coli* may have greater antimicrobial resistance property compared to biofilm non-producing *E. coli.*

Keywords: Multidrug resistant, plasmid, pesticides, PCR

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ABBREVIATIONS

CDC : The Centers for Disease Control and Prevention

CLSI : Clinical and Laboratory Standard Institute Criteria

DAEC : Diffusely adherent *EscherichiaI coli*

EAEC : Enteroaggregative *Escherichia coli*

EHEC : Enterohaemorrrhagic *Escherichia coli*

EIEC : Enteroinvasive *Escherichia coli*

EPEC : Enteropathogenic *Escherichia coli*

EPS : Extracellular polymeric substance

ESBL : Extended-spectrum β-Lactamase

ETEC : Enterotoxigenic *Escherichia coli*

FDA : Food and Drug Administration

HUS : Hemolytic uremic syndrome

MAR : Multiple antibiotic resistance

MBC : Minimum bactericidal concentration

MDR : Multidrug resistant

MIC : Minimum inhibitory concentration

PCR : Polymerase chain reaction

STEC : Shiga-Toxigenic *Escherichia coli*

Stx : Shiga toxin

USDA : United States Department of Agriculture

VTEC : Verocytotoxin-producing *Escherichia coli*

WHO : World Health Organization

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CHAPTER I

INTRODUCTION

1.1 Introduction

Raw salad vegetables are an essential ingredient of a healthy diet. Salad is mainly consumed as a side dish but now has become a major part of food habit in worldwide. Vegetables are essential sources of several nutrients including potassium, dietary fiber, folate (folic acid), vitamin A and vitamin C (USDA 2016). According to World Health Organization (WHO) and Food and Drug Administration (FDA) report a minimum of 400gm of fruits and vegetables per day (excluding potatoes and other starchy tubers) for the prevention of chronic disease such as heart disease, cancer, diabetes, obesity, as well as for the prevention and alleviation of several micronutrient deficiencies, especially in less developed countries (WHO 2018).

Minimally processed food or other raw vegetables have become popular since it suits the present necessity as there is no need of further preparations (Nipa et al 2011). Raw salad vegetables are usually consumed with little or no heat treatment and occasionally without washing and peeling. It may expose the risk foodborne illness to the consumers (Harris et al 2003).

Although, they pose great health benefits, the consumption of fresh vegetables has also been associated with risk for consumers (Weldezgina and Muleta 2016). There has been an increasing case of fresh produces associated foodborne illnesses identified internationally and efforts taking place to resolve these food safety problems (Denis et al 2016).

Consumption of unsafe food containing harmful bacteria, viruses, parasites or chemical substances, causes more than 200 diseases ranging from diarrhea to cancers. An estimated 600 million, almost 1 in 10 people in the world, fall ill after consuming contaminated food and 420,000 die every year, resulting the loss of 33 million healthy life years (WHO 2017).

Contamination of vegetable may take place at all stages during pre and post-harvest techniques (Halablab et al 2011). Using untreated wastewater and manure as fertilizer for the production of vegetables function as major contributing factor to

contamination (Olayemi 1997). Furthermore, possible sources of contamination include soil, feces (human and animal origin), animals (including insects and birds), handling of the product, equipment use in harvesting and processing, and transport (Johannessen et al 2002). Garg et al (2010) affirm that outbreak of infection caused by bacteria, viruses, and parasites have been linked epidemiologically to the consumption of a wide range of vegetables as compared to fruits. Fecal bacteria are able to survive for longer periods in soils (Islam et al. 2004), manure (Kudva et al 1998; Nicholson et al 2005) and water (Steele and Odumeru 2004; Chalmers et al 2000) and thereby act as a potential inoculum for contamination (Holvoet et al 2013).

As a matter of fact, in Nepal the practice of wastewater use in agriculture and in other uses and the emerging environmental and health consequences are not well documented, despite the practice of wastewater irrigation as the antiquated tradition. Also, no guidelines available to ensure safe handling of wastewater and the agricultural produce (Shukla et al 2012).

E. coli is medically important bacteria and is the most prevalent facultative aerobic species in the gastrointestinal tracts of human and animals. Though, it is a harmless microbe but can cause a number of significant illnesses (Friedman et al 2002). *E. coli* is the most common Gram negative pathogen in human that causes urinary tract infections, community and hospital acquired bacteremia (Salvadori et al 2004) and diarrheal diseases (Kaper et al 2004). Thus, antibiotic resistance in *E. coli* is of particular concern.

Drug resistance is increasing gradually due to the overuse of antibiotics, incomplete and under use of medications and widespread practice of feeding livestock with low levels of antibiotics to promote growth (Sultana et al 2014). In such context, raw salad vegetables could serve as vehicle of antimicrobial resistance to pathogenic bacteria from environment to human and from one place to another (Kundu and Islam 2015).

Antimicrobial resistance in *E. coli* has been reported throughout the world. Due to the emergence of resistance to most first-line antimicrobial agents, treatment for *E. coli* has become increasingly complicated (Sabate et al 2008). Over time, resistance to cephalosporins among members of Enterobacteriaceae has increased mostly due to spreading of Extended-spectrum β-Lactamase (ESBL) (Yusha'u et al 2010). Furthermore, resistant *E. coli* are able to transfer antibiotic resistance determinants not

only to other strains of *E. coli*, but also to other bacteria in the gastrointestinal tract and to acquire resistant from other organism (Osterblad et al 2000).

Only a number of documented studies are available showing whether vegetables or the environment where they are produced can act as a carrier or reservoir of antimicrobial resistance (Duffy et al 2005; Ruimy et al 2010).

Biofilms may form on living or inert surfaces and can be established in natural, industrial and hospitalized settings (Hall-Stoodley et al 2004; Lear and Lewis 2012). Biofilms are ubiquitous in nature and has result the clinical and public health microbiologists to study about infectious disease processes from a biofilm. Biofilm-associated microorganisms appear to cause cystic fibrosis, native valve endocarditis, otis media, periodontitis, and chronic prostatitis (Donlan 2001).

Biofilms pose great significance for public health as biofilm-associated microorganisms showed dramatically decreased susceptibility to antimicrobial agents. The antimicrobial susceptibility could be intrinsic (as a natural outcome of growth in the biofilm) or acquired (due to transfer of extrachromosomal elements to susceptible organisms in the biofilm) (Donlan 2001). It is almost impossible to obtain the effective antibiotic minimum bactericidal concentration in-vivo for biofilm eradication by conventional antibiotic administrations (Wu et al 2014).

The study is aim to detect the presence of multidrug resistant *E. coli* in raw salad vegetables and also to know the presence of Shiga-Toxin producing *E. coli* by PCR assay. The study also aims in screening of biofilm producing and biofilm non-producing *E. coli* on raw salad vegetables and their comparative study of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of certain pesticides. Such information will help to expand the present knowledge on drug resistant bacteria on salad vegetables, role of biofilms in antimicrobial resistance property and also help in assessing the potential health risk of Shiga-Toxigenic *E. coli* (STEC) in salad vegetables sold in vegetable markets in Dharan.

1.2 Objectives

1.2.1 General objective

To study about the antibiogram, screening of biofilms, plasmid profiling and occurrence of Shiga Toxin genes of *E. coli* isolated from raw salad vegetables.

1.2.2 Specific objectives

i. To isolate and detect the presence of *E. coli* in raw salad vegetables.
ii. To perform antibiotic susceptibility test by following Kirby-Bauer disc diffusion method in order to know about the antibiotic susceptibility pattern of isolated *E. coli.*
iii. To screen the biofilm formation by *E. coli* isolates using congo red agar method and tube method.
iv. To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of pesticides for biofilm producing and biofilm non-producing *E. coli* isolates by broth microdilution method using 96-well microtiter plate.
v. To perform plasmid profiling of *E. coli* isolates.
vi. To detect the presence of Shiga- Toxin genes of *E. coli* by PCR assay.

CHAPTER II

LITERATURE REVIEW

2.1 Raw salad vegetables

The word ‘salad’ came from the Latin word salata which means salted. A salad containing a wide variety of vegetables such as root vegetables, green vegetables, stalks, stems, and flower is often be closer to a complete meal as compared to many other food possibilities (The World's Healthiest Foods 2018). Fresh salad vegetables and leafy vegetables are ready-to-eat (RTE) produce for healthy and convenient diets and considered as a safe-to-eat by consumers (Lin et al 1996; De Oliveira et al 2011).

Eating any vegetables is beneficial but eating raw vegetables has some additional benefits over cooked vegetables. Vegetables containing higher levels of heat sensitive nutrients such as Vitamin A and C, when cooked at high temperature for longer cooking time degrade or destroy the vitamins. Cooking vegetables in water also cause to lose some water-soluble vitamins, such as folate and Vitamin C, because they leach out into the soaking water (Bruso 2017). These might be the reasons for people to eat salad vegetables in raw condition without proper heat treatment and sometimes not even washing and peeling.

Consuming raw fruits and vegetables has a stronger relationship with better mental health as compared to the consumption of cooked or processed fruits and vegetables (Brookie et al 2018).

Fruit and vegetables are important ingredients of a healthy diet, and their daily consumption at sufficient amount could help prevent major diseases, such as cardiovascular diseases and certain cancers. There are approximately 16 million (1%) disability adjusted life years (DALYs, a measure of the potential life lost due to premature and the years of productive life lost due to disability) and 1.7 million (2.8%) of death worldwide attributed to low fruit and vegetable consumption (WHO 2018).

Furthermore, insufficient intake of fruit and vegetable is estimated to cause around 14% of gastrointestinal cancer deaths, about 11% of ischemic heart disease deaths, about 9% of stroke death globally (WHO 2018).

According to several studies people eating more fruits and vegetables tend to have a higher likelihood of optimal state of mind such as positive emotions (White et al 2013), increased happiness (Lesani et al 2016), life satisfaction (Blanchflower et al 2013; Mujcic and J Oswald 2016), and socio-emotions flourishing with higher openness and extraversion conscientiousness (Conner et al 2017).

Reduced fruit and vegetable intake is associated to poor health and increased risk of non-communicable diseases (NCD). In 2013, an estimated 5.2 million deaths worldwide were attributable to inadequate fruit and vegetable consumption. Consuming fruit and vegetables may also help to prevent weight gain and reduce the risk of obesity, an independent risk-factor for NCDs (WHO 2018).

Apart of being rich sources of vitamins, minerals and dietary fibers fruits and vegetables are also the host of beneficial non-nutrient substances including plant sterols, flavonoids and other antioxidants (WHO 2018).

2.2 Contamination of raw salad vegetables

Fresh vegetables usually carry nonpathogenic epiphytic microorganisms and the produce can become contaminated with pathogens or commensals from human and animal sources during growth and harvesting (Beuchat 2006b; Steele and Odumeru 2004; James 2006).

Raw salad vegetables can become contaminated with pathogenic microorganisms during different means such as while growing in fields, or during harvesting, or in post-harvest handling and also during in processing and distribution. The contamination may occur in various stages during different agronomic practices. Contamination most likely occurs at pre-harvesting stage, either by contaminated manure, sewage, irrigation water, and wastewater from livestock farming or directly from wild and domestic animals. Produce can also be contaminated during harvesting, transport, processing, distribution, and marketing or even at home (Eraky et al 2014; Pagadala et al 2015; Maffei et al 2016).

During harvesting and transportation, raw vegetables may get wounded following in the release of plant nutrients, and thereby, providing substrates for microorganisms present on the surface of the vegetables to grow. Additionally, the processing of fresh salad vegetables may result in altering or increasing the number and types of pathogens present on the surface of the product (Alice 1997).

The majority of microorganisms associated with raw vegetables are Gram negative bacteria likely to dominate the bacterial population (Ngyuen-the and Carlin 1994; Dunn et al 1995; Carno et al 2004). The pathogenic microorganisms residing in intestinal tracts of animals or human are mostly to contaminate vegetables through feces, sewage, untreated irrigation water or surface water (Harris et al 2003). Use of unsafe water for rinsing the vegetables and sprinkling to keep them fresh also acts as a source of contamination (Mensah et al 2002).

The microbial quality of irrigation water is critical as water contaminated with animal or human wastes can introduce pathogens into vegetable products during pre-harvest and post-harvest activities via direct or indirect contaminations (Suslow et al 2003). Therefore, Microbiological quality of irrigation water has an utmost importance to the safety of fresh and minimally processed vegetables (Solomon et al 2002). In addition, Ibenyassine et al (2006) reported that contaminated irrigation water and surface runoff water may be contaminating fruits and vegetables in fields with pathogenic microorganisms.

In developing countries using untreated wastewater and manure as fertilizer for the production of fruits and vegetables plays a major contributing factor to contamination (Amoah et al 2009).

In Nepal, there is almost no documented information available on the practice of wastewater use outside of the Kathmandu valley though farmers in other parts of the country are also known to using wastewater in crop production. The farmers practicing wastewater irrigation use wastewater from various sources including municipal sewage, rivers carrying wastewater and waste stored in the ponds and pools developed in the urban, peri-urban and rural areas (Shukla et al).

2.3 Health risk due to consumption of raw salad vegetables

In contrary to their health benefits, the consumption of fresh fruits and vegetables has also been linked to health risk for consumers (Beuchat 2002a). Many researchers have claimed the vegetables could be associated with outbreak of foodborne illnesses. The size of outbreaks can be different from a few persons to many thousands (Halablab et al 2011).

Pathogens commonly found in salad vegetables include *Staphylococcus aureus, Enterococcus spp., Klebsiella spp., E. coli, Salmonella typhi, Serratia spp., Pseudomonas aeruginosa, Yersinia enterocolitica, Listeria monocytogenes, Aeromonas hydrophila* and *Shigella sonnei* (Viswanathan and Kaur 2001).

E. coli, Clostridium botulinum, Salmonella, L. monocytogenes, Bacillus cereus, and *S. aureus*, able to grow on lettuce, cucumber, carrot, cabbage, tomatoes, and a wide range of other salad vegetables (Abdul-Raouf et al 1993; Lin et al 1996) and have been linked with foodborne illness for decades (Borch and Arinder 2002; Velusamy et al 2010).

During the past few decades the human infections associated with consumption of raw fruits and vegetables have increased (Eraky et al 2014) and have been recognized as a way of transmission of foodborne pathogens (Pagadala et al 2015). The reason behind this increase is the contributing factors which may include changes in agronomic practices and in increase in the number of immune-compromised consumers (Eraky et al 2014).

Every time there is a possibility of *E. coli* O157:H7 being present in vegetables grown in manure fertilized soils (Islam et al 2004). *E. coli* O157:H7, an enterohaemorrhagic strain produces a shiga-like toxin (Griffin and Tauxe 1991; Robinson et al 2006).

The Shiga toxin attacks small blood vessels, destroying intestinal epithelial cells, and causing bloody diarrhea and severe abdominal cramp, along with hemolytic uremic syndrome (HUS), a potentially deadly condition which involve widespread clots in capillaries and hemolytic anemia, thrombocytopenia, and renal failure (Griffin et al 1988; Kaper et al 2004). Treatment could be difficult because antibiotics might increase the risk of HUS. Therefore, treatment is generally limited to the supplement

of fluids, sufficient nutrition, medication for pain and fever, and blood transfusion if necessary (Bitzan 2009; Smith et al 2012).

2.4 Foodborne disease as a global public health concern

Foodborne diseases are a significant factor for morbidity and mortality worldwide. Malnourished infants and children being especially exposed to foodborne hazards and they are at higher risk of developing serious forms of foodborne diarrheal diseases. These, foodborne infections in turn intensify the malnutrition as a result, leading to a vicious circle of debility and mortality (WHO 2015).

People in developing countries often have complication coping with foodborne disease. Also, for large population living at or below the poverty line, foodborne illness cause to continue the cycle of poverty (WHO 2015).

Foodborne illnesses are caused by bacteria, viruses, parasites or chemical substances entering the body via contaminated food or water. Diarrheal diseases are the most common illness resulting from the intake of contaminated foods, causing 550 million people to fall ill and 230,000 deaths every year. Every year 22 million children affected by diarrheal diseases and 96,000 die. Children under five years of age are burdened with 40% of the foodborne disease, with 125,000 deaths every year (WHO 2017).

Each year approximately 800 foodborne disease outbreaks are reported in the U.S., accounting for approximately 15,000 illnesses, 800 hospitalization, and 20 deaths (CDC 2018).

The burden of foodborne diseases is a public health concern globally. Especially, African and South-East Asia regions have the highest incidence and highest death rates, including among children under the age of five years (WHO 2015).

The South-East Asia region has the second highest estimated burden of foodborne disease per population, after the African region. However, regarding of the absolute numbers, more people living in the South-East Asia become ill and die from foodborne diseases every year than in any other region, with more than 150 million cases and 175,000 deaths a year. Approximately 60 million children under the age of

five fall ill and 50,000 die from foodborne diseases in the South-East Asia region every year (WHO 2015).

Despite the growing international awareness of foodborne disease as a significant risk to health and socioeconomic development, food safety still remains overlooked. The complete and original epidemiological data on foodborne disease remain rare, particularly in the developing countries. Several foodborne illnesses remain unrecognized, unreported or uninvestigated (WHO 2015).

2.5 *Escherichia coli*

Escherichia coli (*E. coli*) is a member of Family Enterobacteriaceae that inhabits as a commensal in the intestinal tract of human and animals but sometimes may cause infection in the intestinal tract and other parts of the body. *E. coli* is Gram negative, rod-shaped, non-spore forming, motile with peritrichous flagella or non-motile and measure about 2µm in length and 0.25µm-1µm in width. They are facultative aerobic bacteria (Koneman et al 2005).

E. coli is a major cause of diarrheal diseases, peritonitis, colitis, bacteremia, infant mortality, and urinary tract infections (Russo and Johnson 2003). Some strains may cause cancer too (Arthur et al 2012). Some opportunistic *E. coli* infections are caused by harmless or beneficial strains when introduced to immune-compromised hosts or to parts of a host's body outside of the intestine (Kaper et al 2004). *E. coli* is primarily considered as a community acquired pathogen but it also is the most frequently isolated Gram-negative bacillus found in long term care facilities (Roberts et al 1991).

E. coli strains associated with diarrhea are categorized into six pathotypes and collectively are referred to as diarrheagenic *E. coli* (CDC 2018). They are: Shiga toxin-producing *E. coli* (STEC) or Verocytotoxin- producing *E. coli* (VTEC) or Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC).

EHEC is pathotype causing bloody or non-bloody diarrhea and hemolytic uremic syndrome. A wide variety of food products have been associated with infection. The key virulence factor of EHEC is Shiga toxins also known as Verotoxin which contains

two subgroups stx_1 and stx_2. E. coli O157:H7 is the most important serotype of this group (Nataro and Kaper 1998; Luzader et al 2016). The Shiga toxin is cytotoxic to HeLa (Human cervical cancer cells) and Vero (monkey kidney) cells (Ismaili et al 1995).

ETEC is an important causative agent of diarrhea and death in developing countries and also infects travelers. Clinical symptoms of ETEC appear to be like *Vibrio cholerae* but milder. Specific virulence factor like enterotoxin: heat-stable (ST) enterotoxin and heat-labile (LT) enterotoxin make ETEC distinct from other diarrheagenic *E. coli*. It is able to produce lt_1 and lt_2 or st_a and st_b toxins. lt_2 and st_b are not related to human diseases (Fleckenstein and Rasko 2016; Nataro and Kaper 1998; Kaper et al 2004).

EPEC is another important pathogroup which has been link to diarrhea in children in developing countries. A plasmid called the EAF (EPEC adherence factor) plasmid is possessed by typical EPEC strains encoding a type iv pilus called the bundle-forming pilus (*bfp*). Many types of EPEC have the *eae* chromosomal gene, which encodes the outer membrane protein intimin, and help attaching and effacing lesion on the intestinal mucosa (Nataro and Kaper 1998; Kaper et al 2004; Alikhani et al 2013).

EAEC can cause diarrhea in travelers in both developing and developed countries. This pathogroup affect children and as well as adults. It appears as a new intestinal pathogen causing several outbreaks worldwide. Fimbria structure known as aggregative adhere fimbria (AAFs), which its expression encoded by *aggR* gene, helps EAEC strains adhering to HEp-2 cells and intestinal mucosa. *aggR* gene is positioned in the main virulence plasmids of typical EAEC (p*AA*) (Hebbelstrup Jensen et al 2014; Boisen et al 2008).

EIEC are similar to *Shigella* species in their ability in causing watery diarrhea developing into typical scanty dysenteric stools containing blood and mucus. They are able to invade colonic mucosal cells due to virulence genes present on the pINV plasmid (Nataro and Kaper 1998). They differ from typical *E. coli* as they are non-motile and do not decarboxylate lysine and do not ferment lactose (Feng et al 2016).

DAEC pathotype is less known as compared to other pathotypes. DAEC was initially used to refer to any HEp-2-adherent *E. coli* strain that did not form EPEC-like

microcolonies. This pathotype is now recognized as an independent category of diarrheagenic *E. coli* (Nataro and Kaper 1998). DAEC strains are characterized by a pattern of diffuse adherence (DA), in which the entire cell surface is uniformly covered by the bacteria (Scaletsky et al 1984).

2.5.1 *E. coli* as indicator organism

E. coli is a member of the total coliform group of bacteria and is the only member that can be found in the feces of human and other animals (Health Canada 2006). The presence of *E. coli* can be evidential for recent fecal contamination (WHO 2010). Moreover, *E. coli* cannot survive for long period of time, makes it ideal for identifying recent fecal contamination (Anderson et al 2005; Leclerc et al 2001; Medema et al 2003) and the possible presence of enteric pathogens as well (Geissler et al 2000; US EPA 2002).

2.6 Plasmid

Plasmids are extrachromosomal, self-replicating genetic elements carried by bacteria. A plasmid is defined as a double-stranded circular DNA molecule capable of independent replication. As a matter of fact, plasmids do not carry genes essential for the growth of host cell under non stressful condition (Thomas and Nielsen 2005). They were initially termed as F-factor in *E. coli* even before the double-helix structure of DNA was explained by Watson and Crick (Hayes 1953; Lederberg 1998). The size of plasmid varies from a few to several hundred kilobases and bacterial cells can harbor more than one plasmid species (Tolmasky et al 1992).

Plasmids able to carry and transport genes encoding resistance to antimicrobial compounds. This type of plasmid is predominant in bacteria and can be transferred between different microorganisms, a genetic property that constitutes a very serious medical problem in human and animal medicine. These plasmids, called R plasmid, carry a variety of genes that encode resistance to a wide spectrum of antimicrobial compounds including antibiotics, heavy metals, resistance to mutagenic agents like ethidium bromide and even disinfectant agents such as formaldehyde (Foster 1983; Kummerle et al 1996). R plasmids along with other genetic elements conferring antibiotic resistance can be efficiently maintained and spread within the species by

conjugation, transformation, and transduction (Boyd and Hartl 1997; Houndt and Ochman 2000).

The spread of multiple drug resistance among bacteria is due to plasmids carrying drug resistance phenotypes are known as R-factor. R-factor comprises two components i.e. resistance transfer factor (RTF) and resistance determinant 'r'. The complete plasmid (RTF+ r) is termed as R-factor (Patwary 1994).

The multiple drug resistant bacteria can transfer the antibiotic resistant trait to sensitive bacteria by means of plasmid, resulting in the development of new resistant species or strains (Buxton and Fraser 1997). Besides, these plasmids rapidly dispersed within a bacterial species, and also are highly responsible for the transposition of genes controlling resistance to antibiotics (and other drugs) from one molecule to another (Gardner et al 1991).

Antibiotic resistant *E. coli* may transfer the genes responsible for antibiotic resistance to other species of bacteria such as *S. aureus*, through a process called horizontal gene transfer. *E. coli* bacteria often carry multiple drug resistance plasmids, and under stressful circumstance, readily transfer those plasmids to other species. Mixing of species in the intestine assigns *E. coli* to accept and transfer plasmids from and to other bacteria. Thus, *E. coli* and the other enterobacteria are considered to be important reservoir of transferable antibiotic resistance (Salyers et al 2004).

2.7 Shiga toxins

Shiga toxins are family of a related toxins consist two major groups, stx_1 and stx_2, expressed by genes considered to be part of genome of lamboid prophages (Fried-man and Court 2001). *S. dysentriae* and the STEC are the most common sources of Shiga toxins (Beutin 2006; Spears et al 2006). Shiga toxin (s*tx*) is one of the most potent biological toxins known (Melton-Celsa 2014). The toxin inhibits protein synthesis in eukaryotic cells by removing an adenine residue from the 28S rRNA of the 60S ribosome (Calderwood and Mekalanos 1987; O'Brien et al 1982).

2.7.1 Types of Shiga toxins

There are two distinct immunologically non-cross-reactive *stx* cytotoxins called *stx*$_1$ (or vt$_1$) and *stx*$_2$ (or vt$_2$). STEC strains may manifest *stx*$_1$, *stx*$_2$, both toxins or multiple forms of *stx*$_2$. The *stx*$_1$ toxin is particularly identical to shiga toxin produced by *S. dysenteriae* type I (Nataro and kaper 1998). *stx*$_1$ and *stx*$_2$ are now called *stx*$_{1a}$ and *stx*$_{2b}$ (Scheutz et al 2012). There are two variants of *stx*$_{1a}$: *stx*$_{1c}$ and *stx*$_{1d}$. The *stx*$_{2a}$ subtypes are *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, *stx*$_{2f}$ and *stx*$_{2g}$ (Melton-Celsa 2014).

2.7.2 Structure of Shiga toxins

The Shiga toxin consists of two major subunits, an A (active) subunit (32KDa) and five identical B (binding) subunits (7.7KDa) which form a pentameric ring around the C-terminal of the A subunit (Middlebrook and Dorland 1984; O'Brien and Holmes 1987; Fraser et al 1994). The toxins bind to cellular receptor by the help of B subunits with the internalization of the A subunit which interrupts cell function. The molecular weight of the shiga toxin is approximately 70KDa (Karmali 1989). The sequence identity of *stx*$_1$ and *stx*$_2$ in the A and b subunits are 55% and 57% respectively (Jackson et al 1987).

2.8 Outbreaks of *E. coli* associated with raw salad vegetables

The association of outbreaks of food infections with consumption of ready-to-eat vegetables has been increasing (Pezzoli et al 2008).

As of East Asia, in July 1996, Sakai City, Japan, experienced the largest outbreak of *E. coli* O157:H7 infections ever reported which involved over 7,000 persons (Mermin and Griffin 1999). Michino et al (1999) reported that the outbreak was due to consumption of contaminated white radish sprouts served through a centralized launch program in schools. The report of Ministry of Health and Welfare of Japan (1998) stated that a multiple other outbreaks of *E. coli* O157:H7 infections occurred in Japan during the same summer.

In 2011 there was a serious outbreak of Shiga-toxin producing *E. coli* strain of serotype O104:H4 in Germany. A total of 3816 cases reported on dates ranging from May 1 to July 4. 845 (22 %) of the patients developed the HUS and 54 deaths were

reported. The HUS was prevalent in adults and women were predominant (Frank et al 2011). The enormous rate of HUS cases makes it the largest outbreak of HUS worldwide. Contaminated beans were the vehicle of the outbreak (Burger 2012).

There was a multistate outbreak of STEC O157:H7 infections from ready-to-eat salads in the U.S. in 2013. A total of 33 persons were infected among them 32% of ill persons were hospitalized and two ill persons developed HUS (CDC 2017).

The U.S Food and Drug Administration (FDA) ant the CDC investigated a multistate outbreak of *E. coli* O157:H7 infections likely linked to leafy greens. There were 25 cases in 15 states and the illness onset on dates ranging from November, 2017 to December 12, 2017. Nine were hospitalized, two developed HUS due to complication and one person died. Genetic similarity was found between the bacterial strain isolated from ill persons in the U.S. and the strain of *E. coli* O157 causing an outbreak in Canada during the same time period as the U.S. illness. On December 11, 2017 the Public Health Agency of Canada proclaimed romaine lettuce as the outbreak link (FDA 2018).

There was recently a multistate outbreak of *E. coli* O157:H7 infection linked to romaine lettuce in the U.S. in 2018. A total of 210 people infected, 96 people were hospitalized, 27 people developed HUS and five deaths were reported. The water sample of canal water from Yuma growing region harbored the outbreak strain of *E. coli* O157:H7 which possibly contaminated the romaine lettuce in the region (CDC 2018).

2.9 Antibiotic resistance

The increase in incidence of antimicrobial resistance bacteria in humans, animals and the environment is a major concern in both human and veterinary medicine and is subject to extreme monitoring (Aarestrup 2005; WHO 2000).

It has been observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic varying with time and environment (Hassan 1985) and the popular segments of bacterial population are not the only basis for change in the incidence and levels of antibiotic resistance in natural population and display responses to the

increased exposure of bacteria to antimicrobial compounds over the past several decades (Houndt and Ochman 2000).

Using antimicrobials results an impact on the distribution of antimicrobial resistance phenotypes (Mcgowan and Gerding 1996) and resistant genes (Blake et al 2003). Since, resistant genes may be in mobile genetic elements, resistance to other antimicrobial agents can also be induced by the use of specific antibiotic and can be transferred to a wide variety of bacteria. Resistant plasmids may carry a number of resistance genes and super plasmid encoding resistance to eight and more antimicrobials has been documented (Brock et al 1994).

The use of antimicrobial agents in any environment generates selective pressure that favors the survival of antibiotic pathogens. As stated by the Infectious Diseases Report released by WHO in 2000, drug resistant organisms are prevalent worldwide (WHO 2000).

In recent decades, antimicrobial resistance and reduced sensitivity in pathogenic bacteria have emerged as a serious public health problem in many countries around the world (Rabbi et al 2011).

In addition to prevalence concerns, the global increase of antibiotic resistance in Gram negative bacteria raises critical challenges in healthcare system in treatment of infection by multiple drug resistant bacteria such as *E. coli* O157:H7 with conventional antibiotics inciting the need to investigate new therapeutic approaches (Rahal et al 2012). The prevalence of antimicrobial resistant *E. coli* and possible transmission from soils and the animal husbandry environment to fresh produce at harvest has been documented (Holvoet et al 2013), as has the further flow of resistance from fresh produce bacteria through gene transfer to enterobacterial strains in humans (Bezanson et al 2008).

2.10 Biofilm

Biofilm is a structured consortium formed by microbial cells stick to each other surrounded by the self-produced extracellular polymetric matrix attached on a living or non-living surface. The formation of biofilm is considered an adaptation of microorganisms to antagonistic environments (De la Fuente-Nunez et al 2013; Hall-

Stoodley et al 2004). Biofilms formation by microbes occur due to various different factors such as cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some conditions, by exposure of planktonic cells to sub-inhibitory concentration of antibiotics (Karatan and Watnick 2009; Hoffman et al 2005). A phenotypic shift in behavioral takes place in a cell that switches cell to the biofilm mode of growth and large suites of genes are differentially regulated (An and Parsek 2007).

A range of basic medical devices or other devices used in healthcare environment have turned out to harbor biofilms, resulting in measureable rates of device-associated infections (Stark et al 1999). Several pathogenic bacteria have been associated with, and in some cases, actually grown in biofilms, *Legionella pneumophila* (Murga et al 2001), *S. aureus* (Raad II et al 1992), *Listeria monocytogens* (Wirtanen et al 1996), *E. coli* O157:H7 (Camper et al 1998), *Campylobacter* spp. (Buswell et al 1998), *Salmonella Typhimurium* (Hood and Zottola 1997), *V. cholerae* (Watnick and Kolter 1999) and *Helicobacter pylori* (Stark et al 1999).

Cells attachment is important in the process of biofilm formation. Presence of flagella facilitates attachment of Gram negative bacteria to surfaces (Korber et al 1989). Fimbriae also exhibit importance for attachment (Rosenberg et al 1982). Cell surface hydrophobicity is also an important factor for attachment (Christensen et al 1994).

Biofilm formation takes place by the cells attaching irreversibly to surfaces, cell division begins, forming microcolonies, and producing the extracellular polymers. These extracellular polymeric substances (EPSs) consist basically of polysaccharides that can be detected microscopically and chemical analysis. EPSs form the matrix or structure for the biofilm. They are highly hydrated (98%) water) and firmly bound to the underlying surface. The structure of the biofilm is not just slime of homogenous monolayer but is heterogeneous, both in space and over time, with "water channels" that allow transport of essential nutrient and oxygen to the cells growing within the biofilm (Lewandowski 2000).

2.11 Biofilm and antimicrobial resistance

Bacterial biofilms are distinguished as highly resistant to antibiotic treatment and immune responses (Hoiby et al 2011). Antibiotic treatment is most important and

effective measure for controlling microbial infections, however, antibiotic treatments are almost impossible to eliminate biofilm infections. In-vitro and in-vivo experiments illustrated that the MIC and MBC for bacteria growing as a biofilm were usually much higher (approximately 10-1000 times) than the planktonic bacterial cells (Hoiby et al 2011; Hengzhuang et al 2012).

There are relatively three reasons for the intrinsic antimicrobial resistance of biofilm. First reason is antimicrobial agents diffusing through the EPS matrix to contact and inactivating the organisms within the biofilm. EPSs interrupt diffusion either by chemically reacting with the antimicrobial molecule or by restricting their rate of transport. Second, reduced growth rate of biofilm-associated organisms minimizes the rate of antimicrobial agents taken into the cell and therefore, affect inactivation kinetics. Third, the organisms are further protected by the conditions of environments immediately surrounding the cells within a biofilm. Regarding acquired antimicrobial resistance, research has shown the exchange of plasmids in biofilms under a number of conditions (Donlan 2001).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The materials, equipment, media and reagents used in this study are systematically accounted in Appendix A.

3.2 Methods

The study was conducted at the Microbiology laboratory of Central Campus of Technology, Hattisar, Dharan from March, 2018 to June, 2018.

3.2.1 Study design, study site and sample size

Cross sectional study was done. The samples were collected from three major vegetable markets of Dharan, viz., Krishi Bazar, Sabji mandi, and Palika Tarkari Bazar. A total of 55 samples were analyzed.

3.2.2 Sample collection

Different salad vegetables: carrot, cucumber, cabbage, radish and tomato were collected from the vegetable markets, Dharan. The samples were collected in sterile polythene bags and kept in insulated box with ice and transported to the laboratory without delay.

3.2.3 Laboratory analysis

3.2.3.1 Processing and enrichment of samples

The collected samples were processed and enriched by following method as mentioned in BAM: Diarrheagenic *Escherichia coli* (Feng et al 2016). Firstly the raw salad vegetables were grated into smaller pieces by using sterilized knife and weighed 25gm of the samples aseptically and crushed with the help of mortar and pestle. The vegetable samples were transferred into conical flask containing 225mL of EC broth individually for one sample. The EC broth was incubated at 37^0C for 18-24 hours.

3.2.3.2 Isolation and identification of *E. coli*

After enrichment, one loopful of culture was streaked onto EMB agar plates and incubated at 37^0C for 24 hours. Green metallic sheen colonies on EMB agar were picked and sub cultured onto NA plates. Biochemical tests and gram staining were done for the confirmation of suspected isolates of *E. coli* as according to Bergey's Manual of Systematic bacteriology (2005).

3.2.3.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method on MHA plates using 15 antimicrobial agents: Ampicillin (AMP 10µg), Amoxicillin (AMX 10µg), Amikacin (AK 30µg), Cefazolin (CZ 30µg), Cefoxitin (30µg), Cefotaxime (CTX 30µg), Cefepime (CPM 30µg), Ciprofloxacin (CIP 5µg), Chloramphenicol (C 30µg), Co-Trimoxazole (COT 25µg), Erythromycin (E 15µg), Gentamicin (GEN 10µg), Nalidixic acid (NA 30µg), Nitrofurantoin (NIT 300µg) and Tetracycline (TE 30µg). *E. coli* ATCC 25922 was used as control.

After 18 hours of incubation the results were classified as sensitive, intermediate, or resistant according to the Clinical and Laboratory Standard Institute Criteria (2017). Isolates resistant to three or more than three antibiotic classes were considered as multidrug resistant.

3.2.3.4 Screening of biofilm formation

3.2.3.4.1 Congo red agar method (CRA)

Congo red agar method described by Freeman et al (1989) was performed for screening of biofilm formation by the *E. coli* isolates by using an especially prepared solid medium containing brain heart infusion broth supplemented with 5% sucrose and Congo red stain. The agar plates were inoculated and incubated for 24-48 hours at 37^0C.

Black colonies with a dry crystalline consistency indicated the positive result. Weak slime producers usually remained pink with occasional darkening at the centers of colonies. Darkening of colonies without crystalline morphology indicated

indeterminate result. Experiments were performed in triplicates and repeated three times.

3.2.3.4.2 Tube method (TB)

A qualitative assessment of biofilm formation was performed by using the tube method (Christensen et al 1982). A loopful of microorganism from overnight cultures plates were inoculated into TSBglu (10ml) and incubated for 24 hours at 37^0C. The tubes were decanted and washed with phosphate buffered saline (pH 7.3) and dried. Dried tubes were stained with 0.1% crystal violet solution. After removing excess stain the tubes were washed with deionized water. Washed tubes were then dried in inverted position and observation for biofilm formation was done.

A visible film line on the wall and bottom of the tube was considered positive for biofilm formation. Tubes were examined and the amount of biofilm formation was graded as absent, weak, moderate or strong. The experiment was performed in triplicate and repeated three times.

3.2.3.5 MIC and MBC of pesticides for biofilm producing and biofilm non-producing *E. coli* isolates

The MIC and MBC of pesticides were determined by broth microdilution technique in 96-well microtiter plate. Each well of 96-well plate was aliquoted with 50µL of Mueller Hinton Broth (MHB); 12^{th} well (sterility control) was added with 100µL of MHB. 50µL of pesticide was added into the first well a serial two-fold dilution was performed up to the 10^{th} well; the final 50µL of the suspension was discarded. 0.5McFarland standard broth inoculum was diluted to the ratio of 1:100 and added into 1^{st}-11^{th} well to achieve the final inoculum size at 5×10^5 $CFUmL^{-1}$. Incubation was done at 37°C for 18 hours. Pesticides were tested at the highest concentration of 20% for chlorpyrifos (Insecticides Ltd., India) and 25% for cypermethrin (Plant World Agro Supplier's, Nepal). The lowest concentrations of pesticides with visible signs of no growth/turbidity were considered as the MIC values, whereas the lowest concentrations of pesticides which did not show any bacterial growth when inoculated on MHA plates were considered as MBC values (Chuah et al. 2014). Four of the biofilm producing *E. coli* isolates and four of the biofilm non-producing *E. coli* isolates were comparatively tested.

3.2.3.6 Plasmid profiling

Plasmid DNA extraction was done by small scale alkaline lysis with SDS as described by Sambrook and Russell (2006). Extracted plasmids were electrophoresed by following agarose gel electrophoresis technique using agarose (0.8%) gel stained with ethidium bromide (0.5µg/mL). 10kb DNA ladder (Cleaver Scientific) was used as the DNA standard marker. Plasmid profiles were documented under UV light in gel documentation system.

3.2.3.7 Polymerase chain reaction (PCR) for the detection Shiga-Toxins genes

For the detection of *stx*$_1$ and s*tx*$_2$ gene, PCR of the isolates were performed with the primers listed in Table below.

Chromosomal DNA extraction: The suspected colonies were grown on NA and a loop full of colony was suspended in 100µL of sterile DD water in microfuge tubes. The tube was heated in water bath at 100°C for 10 minutes and rapidly placed on ice for 5 minutes. Then, the tube was centrifuged at 1400 rpm for 3 minutes to precipitate the debris and the supernatant was used as the template DNA.

Primer mix preparation: The 100µM of 10x primer mix was prepared containing 2000nM of each primer in Nuclease free water (Invitrogen).

Table 1: List of the primers

Gene	Primer	Sequence	Size	Reference
stx1	LP30	5' - CAGTTAATGTGGTGGCGAAGG - 3'	348bp	Feng and Monday,2000
	LP31	5' - CACCAGACAATGTAACCGCTG - 3'		
stx2	LP43	5' - ATCCTATTCCCGGGAGTTTACG – 3	584bp	Feng and Monday,2000
	LP44	5' - GCGTCATCGTATACACAGGAGC - 3'		

Table 2: PCR assay setup

Component	Volume (µL)	Concentration
Qiagen Mastermix (2x)	25	1x PCR multiplex mastermix (3mM MgCl2)
10x Primer mix (2 µM of each primer)	5	1x primer mix (0.2 µM of each primer)
Nuclease free water	18	
DNA	2	
Total	50	

For the PCR reaction, the 50 µL PCR mix contain 1x multiplex master mix (Qiagen Hotstar Taq Mastermix, Cat No./ID: 203443), 1x primer master mix, Nuclease free water and 2µL of the template DNA (Table 2) was prepared. The PCR was performed with the following conditions, 95ºC for 15 min for initial activation; then 25 cycles, each cycle consisting of: 95ºC for 1 min, 56ºC for 1 min and 72ºC for 1 min and a 72ºC for 7 min final extension.

Gel electrophoresis was performed in 1% agarose gel with 1x TAE buffer (pH 8.0) for the visualization of DNA and the photograph was taken with the gel documentation system.

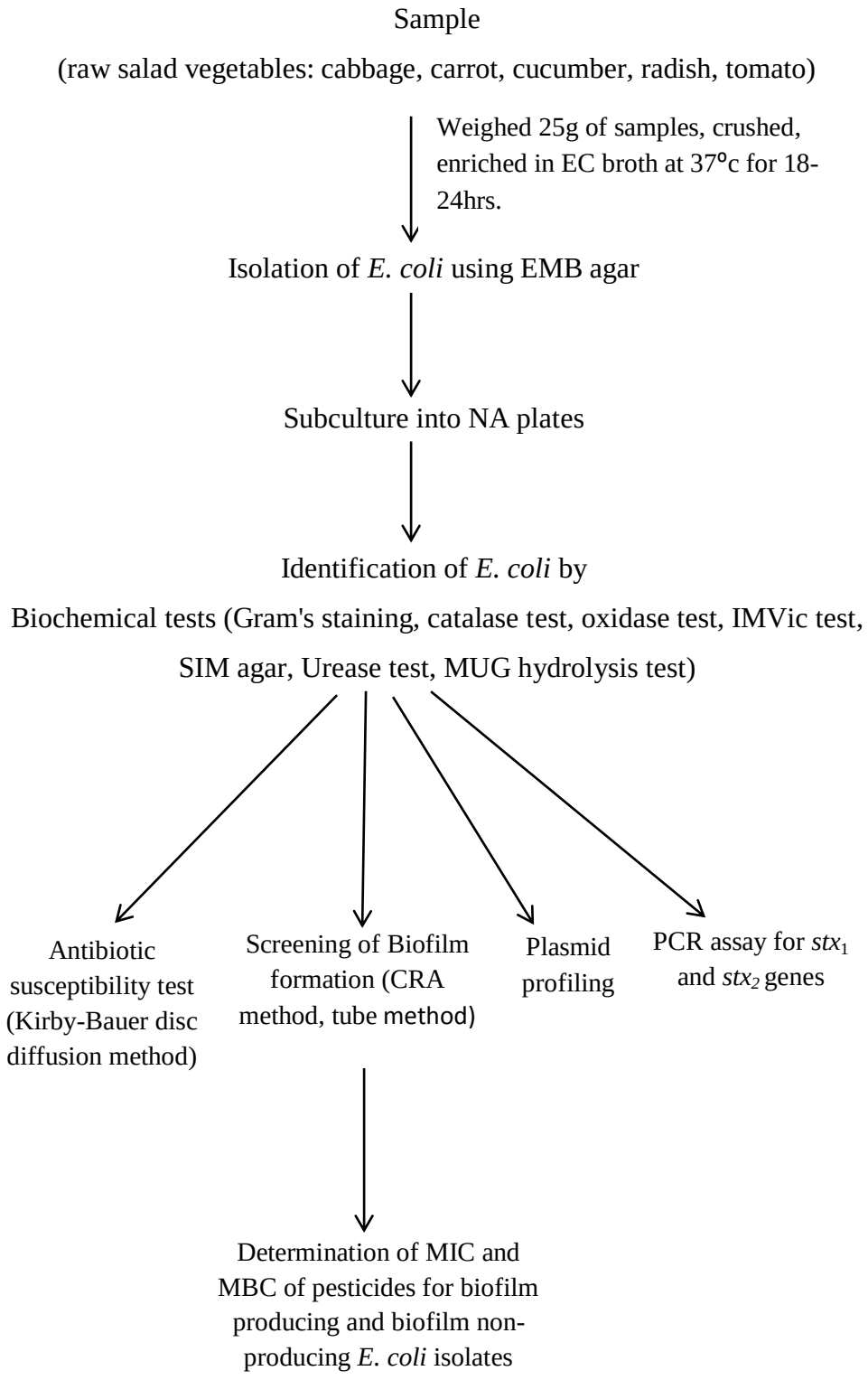

Fig: Flowchart of methodology

CHAPTER IV

RESULTS

A total of 55 samples were analyzed. *E. coli* isolates were subjected for antibiotic profile, detection of biofilm production, determination of MIC and MBC of pesticides (chlorpyrifos 20% and cypermethrin 25%), plasmid profiling and PCR assay for stx_1 and stx_2 genes.

Five different sample types (cabbage, carrot, cucumber, radish and tomato) were collected from three major vegetable markets of Dharan, viz., Krishi bazar, Sabji mandi, and Palika Tarkari bazar. A total of 26 biochemically confirmed *E. coli* were isolated from a total of 55 different raw vegetable salads as listed in Appendix D.

4.1 Prevalence of *E. coli*

E. coli population was noted in 26 (47.27%) of total samples analyzed. The rate of prevalence of *E. coli* was the highest in radish (81.81%) and carrot (63.63%) followed by cucumber (54.54%) and cabbage (27.27%). The value of prevalence was the lowest in tomato (9.09%).

Table 3: Prevalence rate of *E. coli*

Sample / *E. coli*	Cabbage (n=11)	Carrot (n=11)	Cucumber (n=11)	Radish (n=11)	Tomato (n=11)	Total (N=55)
E. coli isolates	3	7	6	9	1	26
Prevalence rate (%)	27.27	63.63	54.54	81.81	9.09	47.27

4.2 Antibiotic susceptibility pattern of *E. coli* isolates

All 26 *E. coli* isolates were subjected for antibiotic susceptibility test using 15 different antibiotics of total nine antibiotic classes. Resistance to one or more antimicrobial agents was found in all of *E. coli* isolates. All isolates of *E. coli* were

completely resistant to erythromycin. The dominant type of resistance was to amoxicillin detected in 25 and ampicillin in 24, followed by cefoxitin in 23, tetracycline in 22, cefotaxime in 20, cefazolin in 19, nitrofurantion in 6, amikacin in 5, chloramphenicol in 1 and co-trimoxazole in 1. The complete antibiotic patterns of all isolates of *E. coli* are listed in Table 4 and Table 5. Other than erythromycin, the higher resistance percentage of *E. coli* isolates was to amoxicillin (96.15%), followed by ampicillin to which 92.31% of *E. coli* isolates were resistant. *E. coli* population was highly sensitive co-trimoxazole (96.15%). *E. coli* isolates showed similar percentage of resistance (3.85%) to chloramphenicol and co-trimoxazole. The complete data of antibiotic susceptibility pattern in percentages is listed in Table 6. All of the isolates were MDR. Multiple drug resistance profile of all MDR *E. coli* isolates are described in Table 7.

Table 4: Antibiotic susceptibility profile

S.N.	Antibiotics	Sample												
		B_{K2}	B_{P2}	B_{S3}	C_{K1}	C_{K2}	C_{P1}	C_{P3}	C_{P4}	C_{S3}	C_{S4}	Cu_{K1}	Cu_{K3}	Cu_{P1}
1	AMP	R	R	R	R	R	R	R	R	R	R	R	R	R
2	AMX	R	R	R	R	R	R	R	R	R	R	R	R	R
3	CZ	I	I	R	R	R	I	R	R	R	R	R	R	R
4	CX	R	R	I	R	R	R	R	R	R	R	R	R	R
5	CTX	I	R	R	R	R	I	R	R	R	R	R	R	R
6	CPM	I	I	I	S	S	S	S	S	S	I	I	I	I
7	GEN	S	S	I	S	S	S	I	S	S	S	S	I	S
8	AK	I	I	R	I	R	R	I	R	I	I	I	I	I
9	TE	I	R	R	R	R	R	R	R	R	R	R	I	R
10	C	S	S	I	I	S	S	I	I	I	I	S	I	S
11	COT	S	S	S	S	S	S	S	S	S	S	R	S	S
12	NIT	R	S	R	S	S	I	I	I	S	S	S	R	R
13	NA	S	S	S	S	S	S	I	S	S	S	I	I	S
14	CIP	S	S	S	S	S	S	S	S	S	S	I	S	S
15	E	R	R	R	R	R	R	R	R	R	R	R	R	R

R=Resistant, I=Intermediate, S=Sensitive; Ampicillin=AMP, Amoxicillin=AMX, Cefazolin=CZ, Cefoxitin=CX, Cefotaxime=CTX, Cefepime=CPM, Gentamicin=GEN, Amikacin=AK, Tetracycline=TE, Chloramphenicol=C, Co-Trimoxazole=COT, Nitrofurantoin=NIT, Nalidixic acid=NA, Ciprofloxacin=CIP, Erythromycin=E

Table 5: Antibiotic susceptibility profile

S.N.	Antibiotics	Sample												
		Cu_{P3}	Cu_{S1}	Cu_{S2}	R_{K1}	R_{K3}	R_{P1}	R_{P2}	R_{P4}	R_{S1}	R_{S2}	R_{S3}	R_{S4}	T_{S3}
1	AMP	R	R	R	I	R	I	R	R	R	R	R	R	R
2	AMX	R	R	R	R	R	R	R	R	R	R	I	R	R
3	CZ	I	I	R	I	R	R	R	R	R	R	R	R	I
4	CX	I	R	R	R	R	R	I	R	R	R	R	R	R
5	CTX	R	R	R	I	R	R	R	I	R	R	S	I	R
6	CPM	I	I	I	S	I	I	S	S	S	S	S	I	I
7	GEN	S	I	S	S	I	S	S	S	S	S	S	S	S
8	AK	I	R	I	I	I	I	I	I	S	S	S	S	I
9	TE	R	I	R	R	R	R	R	R	R	R	I	R	R
10	C	S	S	S	S	S	I	S	S	S	S	S	S	R
11	COT	S	S	S	S	S	S	S	S	S	S	S	S	S
12	NIT	S	I	S	S	I	R	S	S	S	I	S	R	S
13	NA	S	S	S	S	I	S	I	S	S	S	S	S	S
14	CIP	S	I	S	S	I	S	S	S	S	S	S	S	S
15	E	R	R	R	R	R	R	R	R	R	R	R	R	R

R=Resistant, I=Intermediate, S=Sensitive; Ampicillin=AMP, Amoxicillin=AMX, Cefazolin=CZ, Cefoxitin=CX, Cefotaxime=CTX, Cefepime=CPM, Gentamicin=GEN, Amikacin=AK, Tetracycline=TE, Chloramphenicol=C, Co-Trimoxazole=COT, Nitrofurantoin=NIT, Nalidixic acid=NA, Ciprofloxacin=CIP, Erythromycin=E

Table 6: Antibiotic susceptibility profile in percentage

S.N.	Antibiotic	Sensitivity %	Intermediate %	Resistance %
1	Ampicillin	-	7.69	92.31
2	Amoxicillin	-	3.85	96.15
3	Cefazolin	-	26.92	73.08
4	Cefoxitin	-	11.54	88.46
5	Cefotaxime	3.85	19.23	76.92
6	Cefepime	46.15	53.85	-
7	Gentamicin	80.77	19.23	-
8	Amikacin	15.38	65.38	19.23
9	Tetracycline	-	15.38	84.62
10	Chloramphenicol	65.38	30.77	3.85
11	Co-Trimoxazole	96.15	-	3.85
12	Nitrofurantoin	53.85	23.08	23.08
13	Nalidixic acid	80.77	19.23	-
14	Ciprofloxacin	88.46	11.54	-
15	Erythromycin	-	-	100

Table 7: Multiple drug resistance profile

S.N.	Sample	Antibiotics resistant
1	B_{K2}	AMP, AMX, CX, E, NIT
2	B_{P2}	AMP, AMX, CX, CTX, E, TE
3	B_{S3}	AMP, AMX, AK, CZ, CTX, E, NIT, TE
4	C_{K1}	AMP, AMX, CZ, CX, CTX, E, TE
5	C_{K2}	AMP, AMX, AK, CZ, CX, CTX, E, TE
6	C_{P1}	AMP, AMX, AK, CX, E, TE
7	C_{P3}	AMP, AMX, CZ, CX, CTX, E, TE
8	C_{P4}	AMP,AMX,AK,CZ,CX,CTX,E,TE
9	C_{S3}	AMP, AMX, CZ, CX, CTX, E, TE
10	C_{S4}	AMP, AMX, CZ, CX, CTX, E, TE
11	Cu_{K1}	AMP, AMX, CZ, CX, CTX, COT, E, TE
12	Cu_{K3}	AMP, AMX, CZ, CX, CTX, E, NIT
13	Cu_{P1}	AMP, AMX, CZ, CX, CTX, E, NIT, TE
14	Cu_{P3}	AMP, AMX, CTX, E, TE
15	Cu_{S1}	AMP, AMX, AK, CX, CTX, E
16	Cu_{S2}	AMP, AMX, CZ, CX, CTX, E, TE
17	R_{K1}	AMX, CX, E, TE
18	R_{K3}	AMP, AMX, CZ, CX, CTX, E, TE
19	R_{P1}	AMX, CZ, CX, CTX, E, NIT, TE
20	R_{P2}	AMP, AMX, CZ, CTX, E, TE
21	R_{P4}	AMP, AMX, CZ, CX, E, TE
22	R_{S1}	AMP, AMX, CZ, CX, CTX, E, TE
23	R_{S2}	AMP, AMX, CZ, CX, CTX, E, TE
24	R_{S3}	AMP, CZ, CX, E
25	R_{S4}	AMP, AMX, CZ, CX, E, NIT, TE
26	T_{S3}	AMP, AMX, C, CX, CTX, E, TE

4.3 Screening of biofilm formation

In CRA method, only black colonies with dry crystalline morphology was considered as biofilm producer and 18 in 26 isolates tested displayed biofilm positive phenotypes. (Table 8)

A total of 22 isolates obtained as biofilm producers by TM of which ten were strong, seven were moderate and five were weak biofilm producers respectively. (Table 9)

Table 8: Screening of *E. coli* isolates for biofilm formation by CRA method

Sample	Black colonies with dry crystalline morphology	Black/greyish black colonies without crystalline morphology	Pink colonies	Biofilm Formation
B_{K2}	+	-	-	+
B_{P2}	+	-	-	+
B_{S3}	-	-	+	-
C_{K1}	-	+	-	-
C_{K2}	-	-	+	-
C_{P1}	+	-	-	+
C_{P3}	+	-	-	+
C_{P4}	-	-	+	-
C_{S3}	-	+	-	-
C_{S4}	+	-	-	+
Cu_{K1}	+	-	-	+
Cu_{K3}	-	+	-	-
Cu_{P1}	+	-	-	+
Cu_{P3}	-	+	-	-
Cu_{S1}	+	-	-	+
Cu_{S2}	+	-	-	+
R_{K1}	+	-	-	+
R_{K3}	+	-	-	+
R_{P1}	+	-	-	+
R_{P2}	+	-	-	+
R_{P4}	+	-	-	+
R_{S1}	+	-	-	+
R_{S2}	+	-	-	+
R_{S3}	+	-	-	+
R_{S4}	+	-	-	+
T_{S3}	-	-	+	-
Total	18	4	4	18

+ = Positive, - = Negative

Table 9: Screening of *E. coli* isolates for biofilm formation by TM

Sample	Biofilm formation	Biofilm Classification			
		Strong	Moderate	Weak	None
B_{K2}	+	-	+	-	-
B_{P2}	+	-	+	-	-
B_{S3}	-	-	-	-	+
C_{K1}	+	-	-	+	-
C_{K2}	-	-	-	-	+
C_{P1}	+	+	-	-	-
C_{P3}	+	-	+	-	-
C_{P4}	-	-	-	-	+
C_{S3}	+	+	-	-	-
C_{S4}	+	+	-	-	-
Cu_{K1}	+	+	-	-	-
Cu_{K3}	+	-	-	+	-
Cu_{P1}	+	-	+	-	-
Cu_{P3}	+	-	-	+	-
Cu_{S1}	+	+	-	-	-
Cu_{S2}	+	-	-	+	
R_{K1}	+	+	-	-	-
R_{K3}	+	-	+	-	-
R_{P1}	+	+	-	-	-
R_{P2}	+	+	-	-	-
R_{P4}	+	-	+	-	-
R_{S1}	+	+	-	-	-
R_{S2}	+	-	+	-	-
R_{S3}	+	+	-	-	-
R_{S4}	+	-	-	+	-
T_{S3}	-	-	-	-	+
Total	22	10	7	5	4

+ = Positive, - = Negative

4.4 Determination of MIC and MBC

Comparative study of MIC and MBC values of pesticides for biofilm producing and biofilm non-producing *E. coli* isolates was done. Two pesticides: chlorpyrifos 20% and cypermethrin 25% were used. For biofilm producing *E. coli* isolates the MIC and MBC values of chlorpyrifos were at 10% while the MIC and MBC values of cypermethrin were at 12.5% and 25%. In case of biofilm non-producing *E. coli* isolates the MIC and MBC values for cypermethrin were similar i.e. at 3.13% and for chlorpyrifos the MIC value was at 1.25% and the MBC value was at 5%. The values of MIC and MBC of both pesticides for biofilm producing and biofilm non-producing isolates.(Table 10, Table 11, Table 12, Table 13, Table 14, Table 15, Table 16 and Table 17)

Table 10: MIC value of Chlorpyrifos for biofilm producing *E. coli* isolates

Sample	MIC value in%									
	20	10	5	2.5	1.25	0.63	0.32	0.16	0.08	0.04
C_{S3}	-	-	+	+	+	+	+	+	+	+
Cu_{K1}	-	-	+	+	+	+	+	+	+	+
R_{K1}	-	-	+	+	+	+	+	+	+	+
R_{P1}	-	-	+	+	+	+	+	+	+	+

+ = growth, - = no growth

Tables 11: MBC value of Chlorpyrifos for biofilm producing *E. coli* isolates

Sample	MBC value in %									
	20	10	5	2.5	1.25	0.63	0.32	0.16	0.08	0.04
C_{S3}	-	-	+	+	+	+	+	+	+	+
Cu_{K1}	-	-	+	+	+	+	+	+	+	+
R_{K1}	-	-	+	+	+	+	+	+	+	+
R_{P1}	-	-	+	+	+	+	+	+	+	+

+ = growth, - = no growth

Table 12: MIC value of Cypermethrin for biofilm producing *E. coli* isolates

Sample	MIC value in %									
	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05
C_{S3}	-	-	+	+	+	+	+	+	+	+
Cu_{K1}	-	-	+	+	+	+	+	+	+	+
R_{K1}	-	-	+	+	+	+	+	+	+	+
R_{P1}	-	-	+	+	+	+	+	+	+	+

+ = growth, - = no growth

Table 13: MBC value of Cypermethrin for biofilm producing *E. coli* isolates

Sample	MBC value in%									
	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05
C_{S3}	-	+	+	+	+	+	+	+	+	+
Cu_{K1}	-	+	+	+	+	+	+	+	+	+
R_{K1}	-	+	+	+	+	+	+	+	+	+
R_{P1}	-	+	+	+	+	+	+	+	+	+

+ = growth, - = no growth

Table 14: MIC value of Chlorpyrifos for biofilm non-producing *E.coli* isolates

Sample	MIC value in %									
	20	10	5	2.5	1.25	0.63	0.32	0.16	0.08	0.04
B_{S3}	-	-	-	-	-	+	+	+	+	+
C_{K2}	-	-	-	-	-	+	+	+	+	+
C_{P4}	-	-	-	-	-	+	+	+	+	+
T_{S3}	-	-	-	-	-	+	+	+	+	+

+ = growth, - = no growth

Table 15: MBC value of Chlorpyrifos for biofilm non-producing *E. coli* isolates

Sample	MBC value in %									
	20	10	5	2.5	1.25	0.63	0.32	0.16	0.08	0.04
B_{S3}	-	-	-	+	+	+	+	+	+	+
C_{K2}	-	-	-	+	+	+	+	+	+	+
C_{P4}	-	-	-	+	+	+	+	+	+	+
T_{S3}	-	-	-	+	+	+	+	+	+	+

+ = growth, - = no growth

Table 16: MIC value of Cypermethrin for biofilm non-producing *E. coli* isolates

Sample	MIC value in %									
	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05
B_{S3}	-	-	-	-	+	+	+	+	+	+
C_{K2}	-	-	-	-	+	+	+	+	+	+
C_{P4}	-	-	-	-	+	+	+	+	+	+
T_{S3}	-	-	-	-	+	+	+	+	+	+

+ = growth, - = no growth

Table 17: MBC value of Cypermethrin for biofilm non-producing *E. coli* isolates

Sample	MBC value in %									
	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05
B_{S3}	-	-	-	-	+	+	+	+	+	+
C_{K2}	-	-	-	-	+	+	+	+	+	+
C_{P4}	-	-	-	-	+	+	+	+	+	+
T_{S3}	-	-	-	-	+	+	+	+	+	+

+ = growth, - = no growth

4.5 Plasmid profiling

All *E. coli* isolates were subjected for plasmid analysis. Single band of plasmid were observed in 23 isolates, double bands of plasmid were seen in two isolates and one isolates harbored three different sizes of plasmids. The size of plasmids varied from 2.7kb to >10kb. (Table 18)

Table 18: Antibiotic resistance pattern and plasmid profile of *E. coli* isolates

S.N.	Sample	Antibiotic resistance pattern	No. of plasmid	Plasmid size
1	B_{K2}	AMP, AMX, CX, E, NIT	1	>10kb
2	B_{P2}	AMP, AMX, CX, CTX, E, TE	1	>10kb
3	B_{S3}	AMP,AMX,AK,CZ,CTX,E,NIT,TE	1	>10kb
4	C_{K1}	AMP, AMX, CZ, CX, CTX, E, TE	2	3kb, >10kb
5	C_{K2}	AMP, AMX, AK, CZ, CX, CTX, E, TE	1	>10kb
6	C_{P1}	AMP, AMX, AK, CX, E, TE	1	>10kb
7	C_{P3}	AMP, AMX, CZ, CX, CTX, E, TE	1	>10kb
8	C_{P4}	AMP, AMX, AK, CZ, CX, CTX, E, TE	2	3kb, >10kb
9	C_{S3}	AMP, AMX, CZ, CX, CTX, E, TE	3	3kb, 2.7kb, >10kb
10	C_{S4}	AMP, AMX, CZ, CX, CTX, E, TE		
11	Cu_{K1}	AMP, AMX, CZ, CX, CTX, COT, E, TE	1	>10kb
12	Cu_{K3}	AMP, AMX, CZ, CX, CTX, E, NIT	1	>10kb
13	Cu_{P1}	AMP, AMX, CZ, CX, CTX, E, NIT, TE	1	>10kb
14	Cu_{P3}	AMP, AMX, CTX, E, TE	1	>10kb
15	Cu_{S1}	AMP, AMX, AK, CX, CTX, E	1	>10kb
16	Cu_{S2}	AMP, AMX, CZ, CX, CTX, E, TE	1	>10kb
17	R_{K1}	AMX, CX, E, TE	1	>10kb
18	R_{K3}	AMP, AMX, CZ, CX, CTX, E, TE	1	>10kb
19	R_{P1}	AMX, CZ, CX, CTX, E, NIT, TE	1	>10kb
20	R_{P2}	AMP, AMX, CZ, CTX, E, TE	1	>10kb
21	R_{P4}	AMP, AMX, CZ, CX, E, TE	1	>10kb
22	R_{S1}	AMP, AMX, CZ, CX, CTX, E, TE	1	>10kb
23	R_{S2}	AMP, AMX, CZ, CX, CTX, E, TE	1	>10kb
24	R_{S3}	AMP, CZ, CX, E	1	>10kb
25	R_{S4}	AMP, AMX, CZ, CX, E, NIT, TE	1	>10kb
26	T_{S3}	AMP, AMX, C, CX, CTX, E, TE	1	>10kb

4.6 PCR assay for Shiga-Toxin genes

PCR assay was performed for the detection of *stx*$_1$ and *stx*$_2$ genes in all (26) of *E. coli* isolates. A total four of *E. coli* isolates exhibited *stx*$_1$ genes whereas no *stx*$_2$ genes were observed in none of the *E. coli* isolates.

Table 19: PCR assay result

S.N.	Sample	Presence of Shiga-Toxin genes	
		stx$_1$ gene	*stx*$_2$ gene
1	C_{K1}	+	-
2	C_{K2}	+	-
3	C_{P1}	+	-
4	C_{P3}	+	-

+ = present, - = absent

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CHAPTER V

DISCUSSION

Foodborne diseases have been an important issue for all societies (WHO 2015). Factors such as changes in production and processing methods of distribution, consumption patterns, and practices have contributed to increase foodborne infections (Beuchat and Ryu 1997). There has been increasing cases of fresh produces associated foodborne illnesses identified internationally and efforts taking place to resolve these food safety problems (Denis et al 2016).

Raw vegetables can harbor numerous pathogenic microorganisms which may be dispersed over the plants or appear as microcolonies embedded in the plant tissues (Beuchat 2002a). In developing countries, foodborne infections due to consumption of contaminated vegetables are frequent and in some areas they bring about a large proportion of illness as well. However, insufficiency in foodborne disease investigation and surveillance, most outbreaks remain unrevealed and the scientific literature reports only on very few outbreaks (Kundu and Islam 2015).

E. coli along with other different microorganisms grow on lettuce, cucumber, carrot ,cabbage, tomatoes, and a variety of salad vegetables (Abdul-Raouf et al 1993; Lin et al 1996; Velusamy et al 2010) and have been associated with foodborne illness (Borch and Arinder 2002; Velsumay et al 2010). A study by Kim et al (2013) accounted *E. coli* as one of the most common foodborne pathogen.

The use of antimicrobial drugs selects for resistance in pathogenic and as well as in commensal bacteria (van den Bogaard and Stobberingh 2000b). Hence, the commensal population of an individual represents a fine speculation of the selective pressure exerted by the use of antimicrobial agents in that population's environment (van den Bogaard and Stobberingh 1999a). With the help of indicator bacteria such as *E. coli* misinterpretation or overestimation of resistance levels may be minimized (van den Bogaard and Stobberingh 1999a; Lester et al 1990).

In this study five different raw salad vegetables: cabbage, carrot, cucumber, radish and tomato were taken as samples from major three vegetable markets in Dharan. A total of 55 samples were analyzed for the presence of *E. coli* and a total of 26 biochemically confirmed *E. coli* isolates were obtained. The prevalence of *E. coli*

population was found to be 47.27%. Highest number of *E. coli*, nine (81.81%) was isolated from radish, followed by carrot-seven (63.63%), cucumber-six (54.54%), cabbage-three (27.27%) and tomato-one (9.09%).

Similar result was obtained in a study by Nipa et al (2011), in which *E. coli* was isolated in the rate of 40.62% from raw salad vegetables. A little high rate of prevalence was seen in another study done by Rasheed et al (2014) in which a total of 66% *E. coli* were isolated from a total of 150 different food items. Only one isolates of *E. coli* was isolated from tomato samples in the present study with lowest prevalence rate of 9.09%. There was no isolation of *E. coli* from tomatoes by Denis et al (2016) and Nipa et al (2011) but the bacteria was detected on other vegetables such as leafy vegetables, cucumber, carrot etc.

Raw salad vegetables could serve as carriers of antimicrobial resistant pathogens from environments to humans and from the one place to another (Kundu and Islam 2015).

The level of antimicrobial resistance in *E. coli* is helpful indicator of resistance levels assumed in pathogenic bacteria (van den Bogaard and Sobberingh 2000b; Saenz et al 2001). There are several studies documenting the drug resistant *E. coli* and other coliforms in vegetables (Osterblad et al 1999), egg (Arathy et al 2011), milk (Cizek et al 2008) and raw meat (Srinivasa Rao et al 2011).

All isolates were resistant to at least four or more antibiotics. All of the isolates were MDR resulting in prevalence rate at its highest peak (100%). The MDR *E. coli* isolates were resistant to at least four to eight antibiotics. Among all MDR isolates, a total of 16 isolates were resistant to five different antibiotic groups, eight were resistant to five different antibiotic groups, one was resistant to six different antibiotic groups and one was resistant to three different antibiotic groups. Similarly, a study by Adeshina et al (2012), 75% of the *E. coli* isolates showed multiple antibiotic resistance. A different result was obtained by Rasheed et al (2014) in India, in which overall incidence of drug resistant *E. coli* was 14.7%. According to a recent study by Faour-Klingbeil et al (2016) the distribution of MDR *E. coli* isolated from different points on the fresh salad and vegetable supply chain in Lebanon was found to be 60%.

A total of 15 antibiotics were used against the all 26 isolates. All isolates were resistant to erythromycin. Besides, erythromycin *E. coli* isolates were highly resistant

to amoxicillin (96.15%), followed by ampicillin (92.31%), cefoxitin (88.46%), tetracycline (84.62%), cefotaxime (76.92%), cefazolin (73.08%), nitrofurantoin (23.08%) and amikacin (19.23%). Resistance to chloramphenicol and co-trimoxazole were similar which was 3.85%. All *E. coli* isolates were sensitive to intermediate against cefepime, gentamicin, nalidixic acid and ciprofloxacin. Co-Trimoxazole was the most sensitive antibiotic (96.15%), followed by ciprofloxacin (88.46%) against the isolates whereas, gentamicin and nalidixic acid shared similar sensitivity percentage (80.77%).

Similar result of resistivity of *E. coli* against erythromycin was documented by Nipa et al (2011) and ampicillin was also completely resistant in that result but the present study showed ampicillin as the third highest resistant antibiotic to isolates. Faour-Klingbeil et al (2016) isolated tetracycline resistant *E. coli* at nearly similar percentage (80%) with the present study (84.62%). In their study, more than two-thirds of MDR *E. coli* (69%) showed resistance to ampicillin and tetracycline but the present study reported higher level of resistivity of ampicillin (92.31%) and tetracycline (84.62%). A similar result with the present study of resistivity of chloramphenicol (4.6%) was documented by Rasheed et al (2014).

However, conflicting results were reported by Adeshina et al. (2012) in which the *E. coli* isolated from vegetable salad samples were 100% sensitive to nalidixic acid and in the present study it is 80.77%. The results varied with the present study for the resistance to antibiotics like nitrofurantioin (75%), gentamicin (50%), co-trimoxazole (11.3%) ciprofloxacin (6.6%) and cefotaxime (5.3%).

In plasmid profiling all *E. coli* isolates harbored plasmid DNA to different sizes (2.7kb to >10kb). All those isolates were MDR and possessed at least single plasmid. Large numbers of plasmids were of size of greater than 10kb. Two isolates harbored double plasmids of sizes of approximately 3kb and >10kb where as one isolate possessed three different plasmids of sizes of approximately 2.7kb, 3kb and >10kb.

As a matter of fact, Alam et al (2013) showed a significance correlation between antibiogram and plasmid profiling; all isolates of *E. coli* harbored plasmid (6kb-33kb) and all the isolates showing resistance to antibiotics (one or more antibiotics) harbored one or more plasmids. Also, in the study conducted in pediatric diarrhea by Babu et al (2009), approximately 64% of *E. coli* isolates harbored plasmids, 76.1% of

them were able to transfer their plasmids, and *E. coli* strains with plasmids were more resistance to certain antibiotics than those without plasmids.

Correspondingly, plasmid profiling by Khadgi et al. (2013) showed that almost all MDR *E. coli* isolates contained plasmids and many of them shared some common plasmids too but there was no relation detected between the antibiotic resistance pattern and the plasmid profile analysis.

As reported by Christopher et al (2013), *E. coli* isolates with high MAR profiles were found to possess multiple plasmids (2-23kb) and showed that antibiotic resistance is controlled by the plasmid number and plasmid size. In contrast to the present study; although all MDR *E. coli* isolates exhibited plasmids, the isolates harboring multiple plasmids showed synonymous MAR profiles with some of isolates containing single-sized plasmid.

PCR assay was done for all of *E. coli* isolates for the detection of Shiga-toxin genes (stx_1 and stx_2 genes). stx_1 genes were observed in total of four (15.38%) *E. coli* isolates, all were isolated from carrots. Other than carrot, stx_1 genes were not detected in any other vegetables. There was no presence of stx_2 gene in any of the vegetable samples analyzed.

In similar type of study done by Rasheed et al 2014, out of 31 STEC positive strains, eight (25.8%) exhibited only stx_1 gene isolated from raw egg outer surface, raw sheep meat and hand-washing water from street vendors), 17 (54.8%) exhibited both stx_1 and stx_2 genes isolated from raw chicken, vegetable salad, raw sheep meat, raw egg surface and unpasteurized milk. Another study showed 11.6% meat samples and 1.8% vegetable samples presumptively contaminated by STEC. Among vegetable samples, 1.5% samples were positive for stx_1 and/or stx_2 genes (Bardasi et al 2015).

In screening of biofilm production by *E. coli* isolates, two methods were performed: CRA method and TM. CRA method showed 18 positive biofilm producing *E. coli* isolates whereas TM displayed 22 positive biofilm producing *E. coli* isolates. As the TM tends to give more sensitive, specific and accurate result than CRA method (Mathur et al 2006), the result displayed by TM was considered as the final result of screening of biofilm formations. Thus, 22 (84.62%) of E. coli isolates were biofilm producers.

There is association of biofilms with emergence of antibiotic resistant bacteria (Chadha 2014). The high resistance nature of biofilms may be due to several mechanisms that have been explored and considered to be key factors. The mechanisms are: limited diffusion, enzyme causing neutralizations, heterogeneous functions, slow growth rate, presence of persistent (non-dividing) cells and biofilm phenotype such adaptive mechanisms e.g. efflux pump and membrane alteration (Hogan and Kolter 2002; Poole 2002).

The phenotypic conversion from a free-swimming, planktonic form to a sessile existence in a biofilm is an extremely regulated developmental process that depends on numerous environmental and genetic factors which vary from species to species (O'Toole et al 2005; Monds and O'Toole 2009; López et al 2010). In *E. coli* and *P. aeruginosa* exposure to sub-inhibitory concentrations of aminoglycosides is one of the many signals that can initiate biofilm formation (Hoffman et al 2005). Larson et al (2003) stated that the thickness of *E. coli* biofilms may be the size of hundreds of microns and causes a difficulty in treatment with antibiotics due to presence of exopolymers.

Pathogenic bacteria in a biofilm employ both tolerance and resistance mechanisms to withstand antimicrobial challenges. However, there appears to be some disagreement in the literature as some authors might claim a particular mechanism confers resistance, other might state it as a tolerance mechanism (Hall and Mah 2017). Likewise, in the present study the MIC and MBC of pesticides (chlorpyrifos 20% and cypermethrin 25%) showed that higher concentration of both pesticides were required for the killing of biofilm producing *E. coli* isolates as compared to biofilm non-producing *E. coli* isolates which indicated that bacteria residing in biofilms tend to have greater antimicrobial resistance property.

A report was presented by Ito et al (2009) about increased antibiotic resistance of *E. coli* in mature biofilms and stated that ofloxacin and kanamycin were effective against biofilm cells but ampicillin did not kill the cells in biofilm, resulting in the regrowth of biofilm after the ampicillin treatment was discontinued.

The presence of *E. coli* in raw salad vegetables in the present study indicated the contamination of the salad vegetables by the bacteria. The raw salad vegetables might have become contaminated during agronomic practices or by unhygienic handling

during marketing. Also in the study, the prevalence of MDR *E. coli* was at its topmost rate implying the emergence of MDR bacteria in salad vegetables in hideous manner. Plasmid profiling showed the presence of plasmid in all MDR isolates. For the confirmation of plasmid origin and MDR pattern further study on the isolates such as plasmid curing could be done. The presence of stx_1 genes in *E. coli* isolates revealed the risk of foodborne illnesses by the Shiga toxins. Also, biofilm producing *E. coli* isolates were more resistant to pesticides tested as compared to biofilm non-producing *E. coli* isolates.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In conclusion, the salad vegetables sold in all major three vegetable markets in Dharan are contaminated with *E. coli.* The high presence of MDR *E. coli* in raw salad vegetables indicates the possible outbreak of associated organism and difficulty in their treatment. The presence of STEC in carrots indicates the possible STEC infections to the consumers. Biofilm producing *E. coli* may sustain their selves more compared to biofilm non-producers against pesticides.

5.2 Recommendation

1. Presence of *E. coli* in salad vegetables indicated the recent fecal contamination and presence of other possible enteric pathogens.
2. Since, MDR *E*. coli isolates were high the monitoring of antibiotic profile of *E. coli* is needed.
3. Since, salad vegetables were highly contaminated with MDR *E. coli* proper washing, peeling of salad vegetables before consumption is must.
4. Biofilms may help the microorganisms in resisting antimicrobial agents.
5. Further study on presence of other MDR bacteria in salad vegetables can be done.
6. Regular surveillance of antibiotic pattern of bacteria is important.

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APPENDIX A

List of materials

1. Equipment/Instrument

1. Autoclave
2. Hot air oven
3. Incubator
4. Electrical balance
5. Laminar air flow cabinet
6. Microscope
7. Micropipette
8. Horizontal gel documentation apparatus
9. Refrigerator
10. Water shaker bath
11. Cold centrifuge
12. Vortex machine
13. Microwave oven
14. Gel documentation system

2. Microbiological/biochemical media

1. Nutrient agar (HiMedia)
2. Nutrient broth (HiMedia)
3. EMB agar (HiMedia)
4. SIM medium (HiMedia)
5. MR-VP broth (HiMedia)
6. Simmons citrate agar (HiMedia)
7. Urea agar base (HiMedia)
8. Urea (HiMedia)
9. MUG EC O157 agar (HiMedia)
10. Tryptone soy broth (HiMedia)
11. Mueller Hinton agar(HiMedia)
12. Mueller Hinton broth (HiMedia)
13. Peptone (HiMedia)
14. Agar-agar (MERCK)
15. Agarose (HiMedia)
16. Brain heart infusion agar (HIMedia)
17. Sucrose (HiMedia)
18. Lactose (HiMedia)
19. Tryptose (HiMedia)
20. Bile salt (HiMedia)

3. Chemicals/Reagents

1. Lysol
2. Gram's iodine
3. Crystal violet
4. Safranin
5. Ethyl alcohol
6. Oxidase reagent
7. Hydrogen peroxide
8. Methyl red solution
9. Alpha-napthol solution
10. Kovac's reagent
11. Sodium chloride
12. Dipotassium hydrogen phosphate
13. Potassium dihygrogen phosphate
14. Congo red stain
15. Ethidium bromide (HiMedia)
16. Conc. Sulfuric acis
17. Barium chloride

4. Glassware

1. Test tube
2. Petriplates

3. Conical flask
4. Measuring cylinder
5. Glass rod
6. Glass slide
7. Beaker

5. Extraction/lysis buffer and solution

1. Alkaline lysis solution I

Components	Volume (mL)
1M glucose	5
1M Tris-Cl	2.5
0.5M EDTA	1
De-ion water	90.5
Total volume	100

2. Alkaline lysis solution II

Components	Volume (mL)
0.2N sodium hydroxide	2
1% SDS	10
De-ion water	88
Total volume	100

3. Alkaline lysis solution III

Components	Volume (mL)
5M potassium acetate	60
Glacial acetic acid	11.5
De-ion water	28.5
Total volume	100

4. 10X Tris EDTA (TE) – Stock solution (working solution- 1X)
100mM Tris-Cl (pH 8.0)
10mM EDTA (pH 8.0)

5. 50X TAE – Stock solution (working solution- 1X)

Tris base 242gm
Glacial acetic acid 51.7ml
0.5M EDTA 100ml (pH 8.0)
Distilled water 700ml

6. Et-Br
1gm of Et-Br to 100ml of water

7. Gel loading dye (6X)
0.25% (w/v) bromophenol blue, 40% (w/v) sucrose

6. PCR reagents

Qiagen Hotstar Taq Mastermix (2X) (Qiagen Cat No. /ID: 203443)
Forward primer (Sigma)
Reverse primer (Sigma)

Nuclease free water (InVitrogen)

7. DNA ladder:10kb, 3kb (Cleaver Scientific)

8. Miscellaneous

1. Aluminum foils
2. Immersion oil
3. Cotton
4. Microfuge tubes
5. Inoculating loop
6. Labeling tape
7. Blotting paper
8. Tissue paper
9. Pesticides: a) Chlorpyrifos 20% EC [Insecticides (India) Ltd]
   b) Cypermethrin 25% E.C. (Plant World Agro Supplier's)
10. Spatula
11. Forceps
12. Test tube stand
13. 96-well plate
14. Cotton swabs
15. Samples
16. Micropipette tips
17. Antibiotic disc

9. Standard organism

Escherichia coli ATCC 25922

APPENDIX B

Methodology of biochemical tests for identification of *E. coli*

1. Catalase test

2-3 drops of 3% H_2O_2 was poured onto a clean glass slide, and then a small amount of organism from NA culture plate was added to it and mixed well. The bubble formation indicates the positive test reaction.

2. Oxidase test

A little amount of organism from NA plate culture was transferred to oxidase plate. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

3. Gram's staining

A clean glass slide was taken and a thin smear of test organism was made on it and air dried. The smear was then heat fixed. Crystal violet was added to the smear for 30 sec. and washed with distilled water. The smear was covered with Gram's iodine for 60 sec. and washed off with 95% ethanol. It was washed with distilled water and drained. Safranin was applied to smear for 30 sec. and washed with distilled water. The stained slide was set to air dry. The slide was examined microscopically using oil-immersion objective.

4. Indole test

1% of tryptone broth was prepared and autoclaved. Test organism was inoculated into tryptone broth and incubated at 37°C for 24 hours. After incubation, 1ml of Kovac's reagent was added to the tubes. The tubes were shaken gently after intervals of 10-15 minutes. The tubes were allowed to stand to permit the reagent to come to the top. Development of a cherry red colour in the top layer of the tubes indicates a positive test.

5. MR-VP test

MR-VP broth was prepared and sterilized. The MR-VP tubes were then inoculated with test organism and incubated at 37°C for 24 hours. For MR test, 5 drops of methyl red indicator was added to the tubes, and the development of red coloration indicates the positive test. For VP test, 12 drops of VP reagent I and 2-3 drops VP reagent II were added to the tubes and shaken gently for 30 sec. with the caps off to expose the media to oxygen. The reaction was allowed to complete for 15-30 mins. The development of crimson to ruby pink colour is indicative of positive VP test.

6. Citrate Utilization test

Simmons citrate agar was sterilized and slants were prepared. The slants were inoculated with test organism, by means of a stab-and-streak inoculation. The tubes were incubated at 37°C for 24 hours. In a positive test, the colour of medium changes from green to blue.

7. SIM agar

SIM agar medium was prepared. The test organism was inoculated into the SIM agar deep tubes by means of stab inoculation. The inoculated tubes were inoculated at 37°C for 24 hours. Blackening of the culture medium indicates the H_2S production, on addition of Kovac's reagent there will be development of cherry red layer on the top surface of tubes indicates the positive indole test and A positive motility test is indicated by a diffuse zone of growth flaring from the line of inoculation.

8. Urease test

Urea agar base was sterilized and cool to 50°C, 40% of sterile urea solution of appropriate volume was added to it. Urea agar slants were prepared and inoculated with test organism. The tubes were then incubated at 37°C for 24 hours. Deep pink coloration of the medium indicates positive reaction for the degradation of urea by production of an enzyme urease.

9. MUG hydrolysis test

MUG EC O157 agar medium was prepared and inoculated with test organism. The inoculated petriplates were incubated at 37°C for 24 hours. After incubation, the culture plates were examined under UV light. Presence of growth and production of a bright yellow fluorescence is considered positive for the hydrolysis of MUG by *E. coli*.

APPENDIX C

Zone size interpretative chart of Antibiotic Susceptibility Test

Antibiotics	symbol	Disc content (µg)	Diameter of zone of inhibition in mm		
			Sensitive	Intermediate	Resistant
Ampicillin	AMP	10	17	14-16	13
Amoxicillin	AMX	10	17	14-16	13
Cefazolin	CZ	30	23	20-22	19
Cefoxitin	CX	30	18	15-17	14
Cefotaxime	CTX	30	26	23-25	22
Cefepime	CPM	30	25	19-24	18
Gentamicin	GEN	10	15	13-14	12
Amikacin	AK	30	17	15-16	14
Tetracycline	TE	30	15	12-14	11
Chloramphenicol	C	30	18	13-17	12
Co-Trimoxazole	COT	25	16	11-15	10
Nitrofurantoin	NIT	300	17	15-16	14
Nalidixic acid	NA	30	19	14-18	13
Ciprofloxacin	CIP	5	21	16-20	15

(Source: Product information guide: HiMedia Laboratories Pvt. Limited, India)

APPENDIX D

Sample distribution

Sample location	Sample type					*E. coli*
	Cabbage	Carrot	Cucumber	Radish	Tomato	
K	B_{K1}	C_{K1}	Cu_{K1}	R_{K1}	T_{K1}	C_{K1}, Cu_{K1}, R_{K1}
	B_{K2}	C_{K2}	Cu_{K2}	R_{K2}	T_{K2}	B_{K2}, C_{K2}
	B_{K3}	C_{K3}	Cu_{K3}	R_{K3}	T_{K3}	Cu_{K3}, R_{K3}
P	B_{P1}	C_{P1}	Cu_{P1}	R_{P1}	T_{P1}	C_{P1}, Cu_{P1}, R_{P1}
	B_{P2}	C_{P2}	Cu_{P2}	R_{P2}	T_{P2}	B_{P2}, R_{P2}
	B_{P3}	C_{P3}	Cu_{P3}	R_{P3}	T_{P3}	C_{P3}, Cu_{P3}
	B_{P4}	C_{P4}	Cu_{p4}	R_{P4}	T_{P4}	C_{P4}, R_{P4}
S	B_{S1}	C_{S1}	Cu_{S1}	R_{S1}	T_{S1}	Cu_{S1}, R_{S1}
	B_{S2}	C_{S2}	Cu_{S2}	R_{S2}	T_{S2}	Cu_{S2}, R_{S2}
	B_{S3}	C_{S3}	Cu_{S3}	R_{S3}	T_{S3}	$B_{S3}, C_{S3}, R_{S3}, T_{S3}$
	B_{S4}	C_{S4}	Cu_{S4}	R_{S4}	T_{S4}	C_{S4}, R_{S4}

K=Krishi Bazar, P=Palika Tarkari Bazar, S=Sabji mandi; B=cabbage, C=Carrot, Cu=Cucumber, R=Radish, T=Tomato

PHOTOGRAPHS

Photograph 1

Fig: Green metallic sheen of *E. coli* on EMB agar

Photograph 2

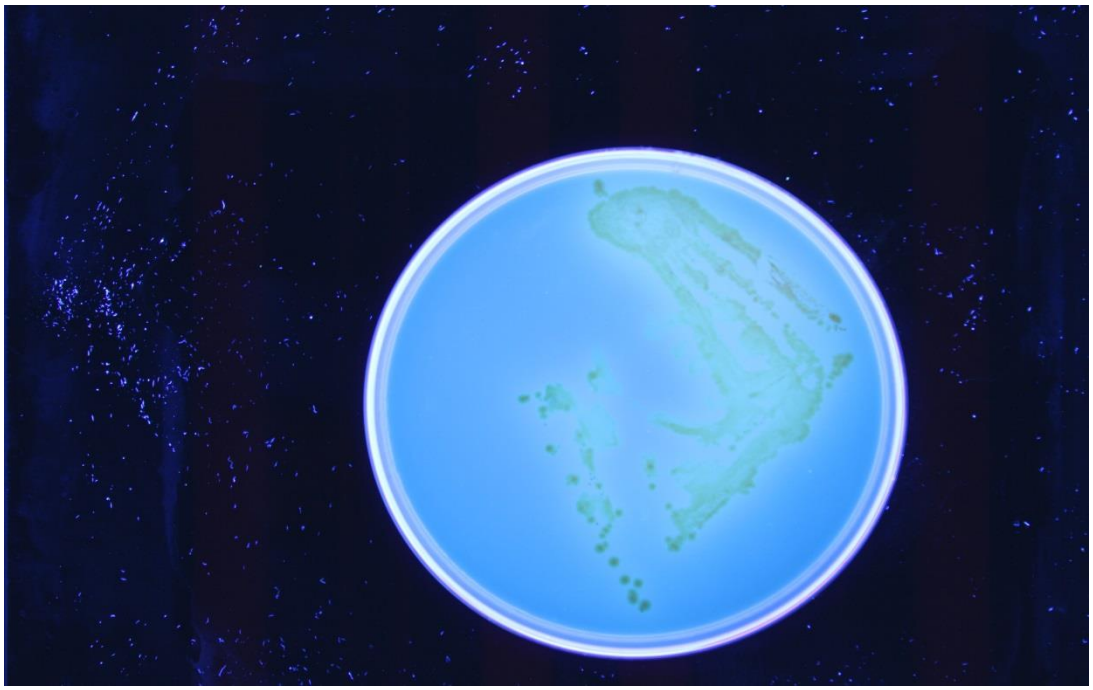

Fig: Fluorescent *E. coli* colonies on EC-MUG agar plate under UV light

Photograph 3

Fig: Black colonies with dry crystalline morphology of *E. coli* on CRA

Photograph 4

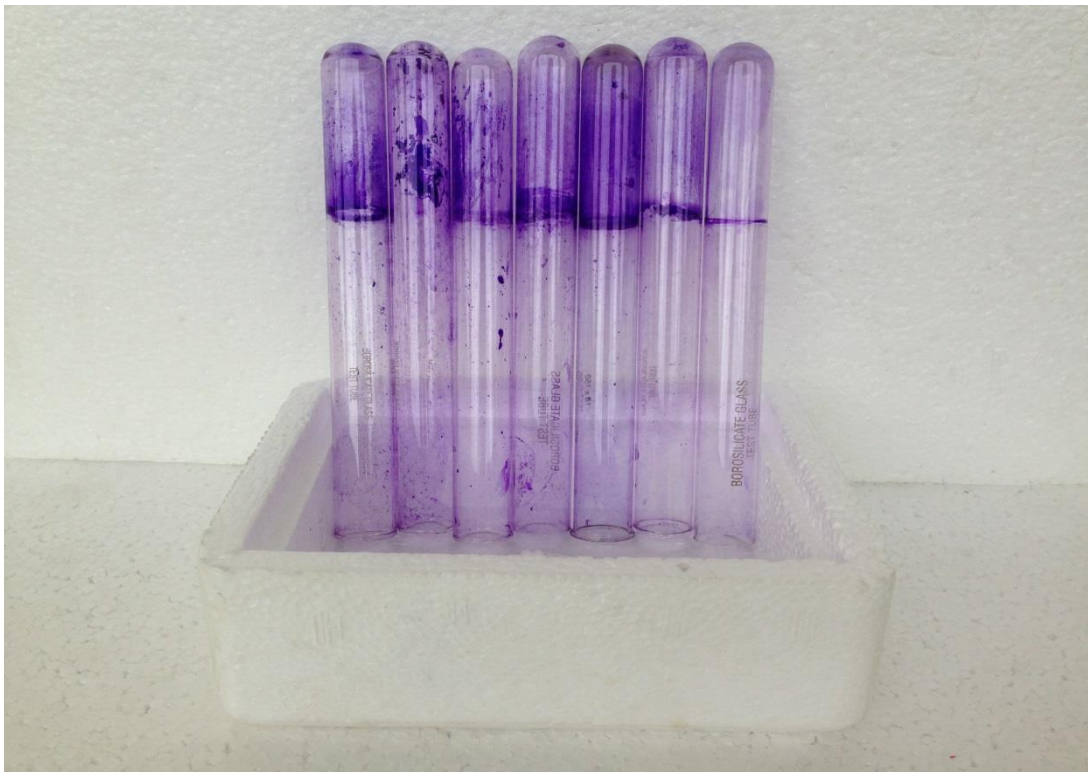

Fig: Screening of biofilm formation by TM

Photograph 5

Fig: Antibiotic susceptibility test

Photograph 6

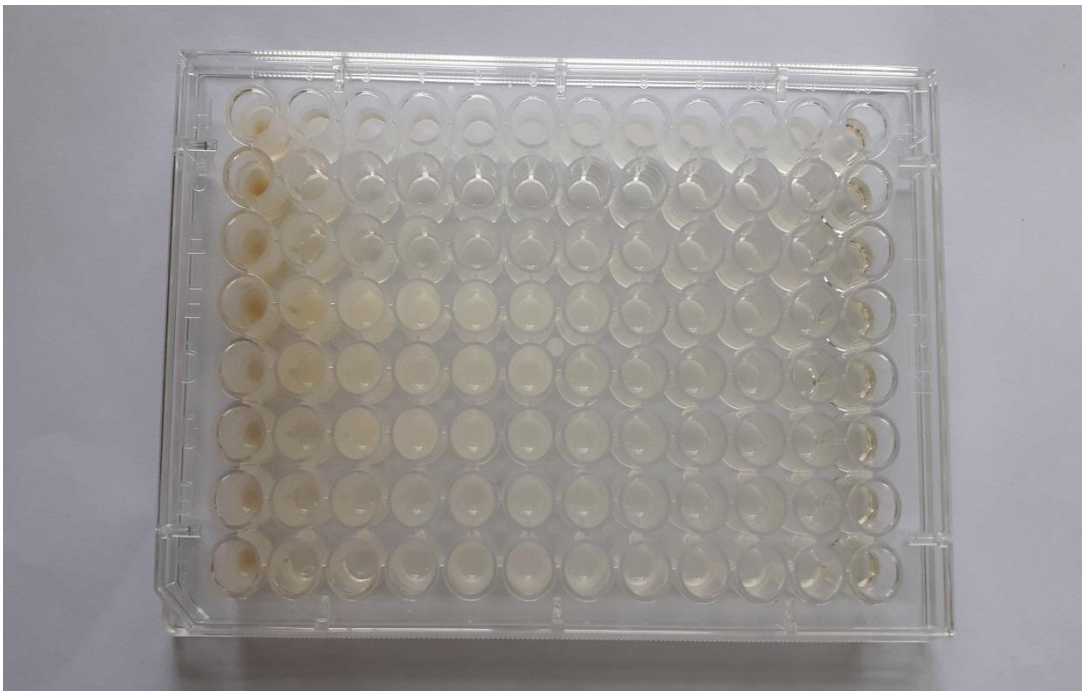

Fig: Determining MIC of pesticides by 96-well microtiter plate method

Photograph 7

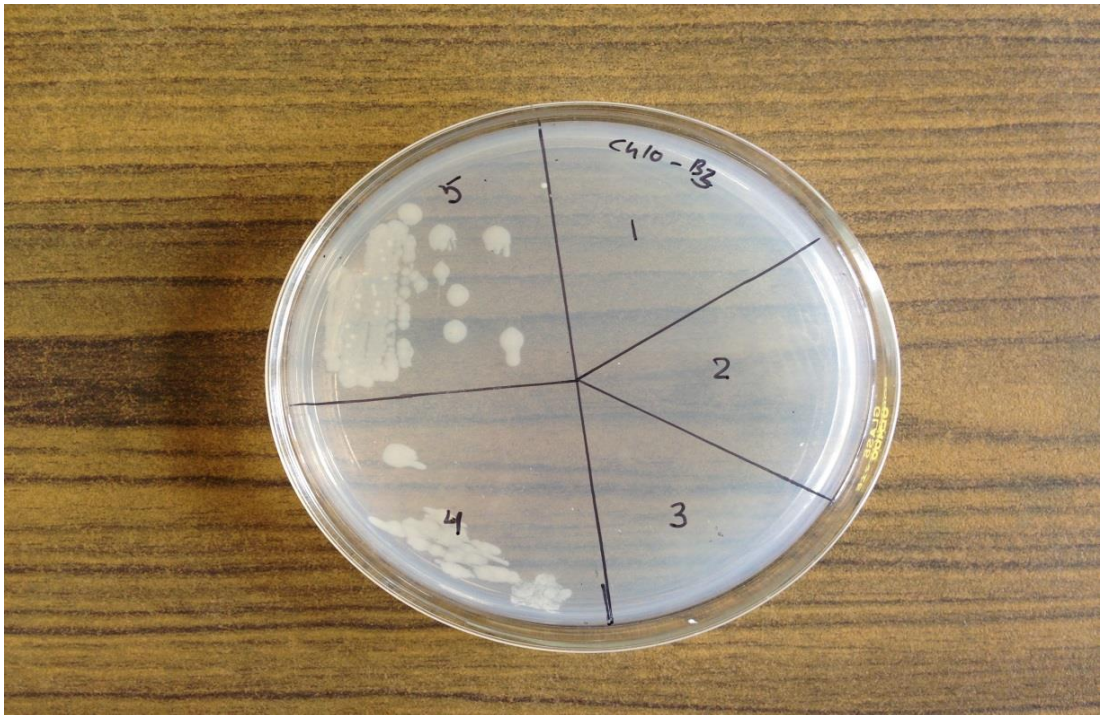

Fig: MBC of Chlorpyrifos 20% for biofilm non-producing *E. coli*; sample: B_{S3}; concentrations: 1, 2, 3, 4, 5: 20%, 10%, 5%, 2.5%, 1.25%

Photograph 8

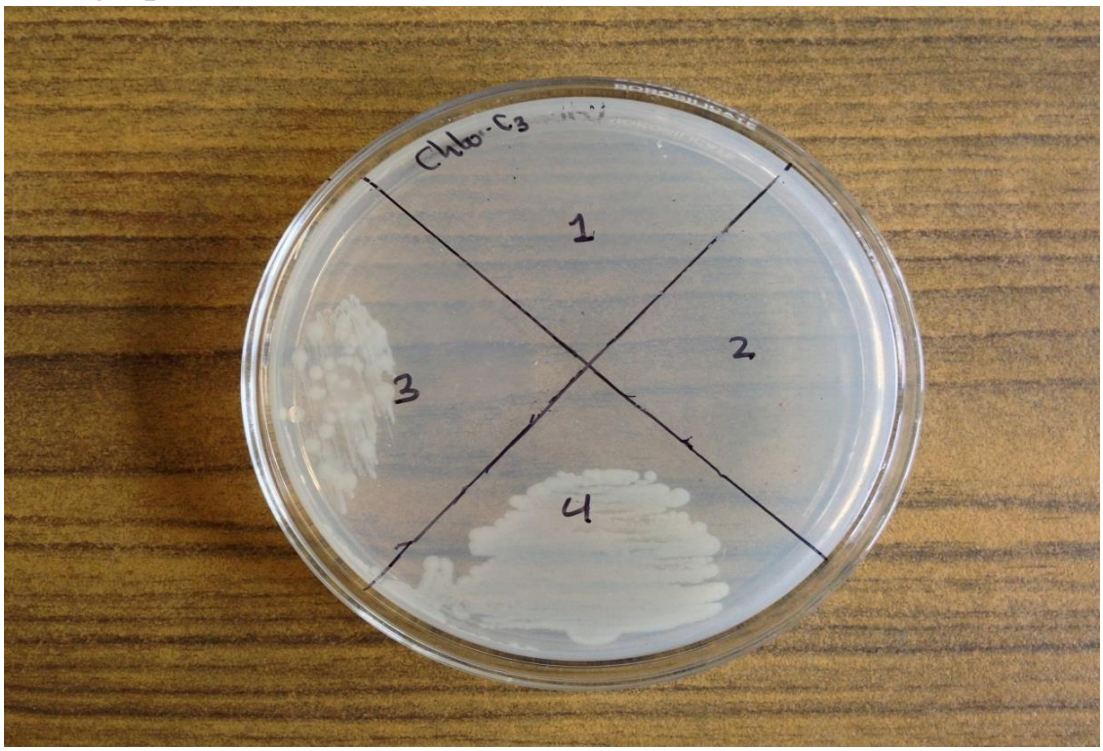

Fig: MBC for Chlorpyrifos 20% for biofilm producing *E. coli*; sample: C_{S3}; concentrations: 1, 2, 3, 4: 20%, 10%, 5%, 2.5%

Photograph 9

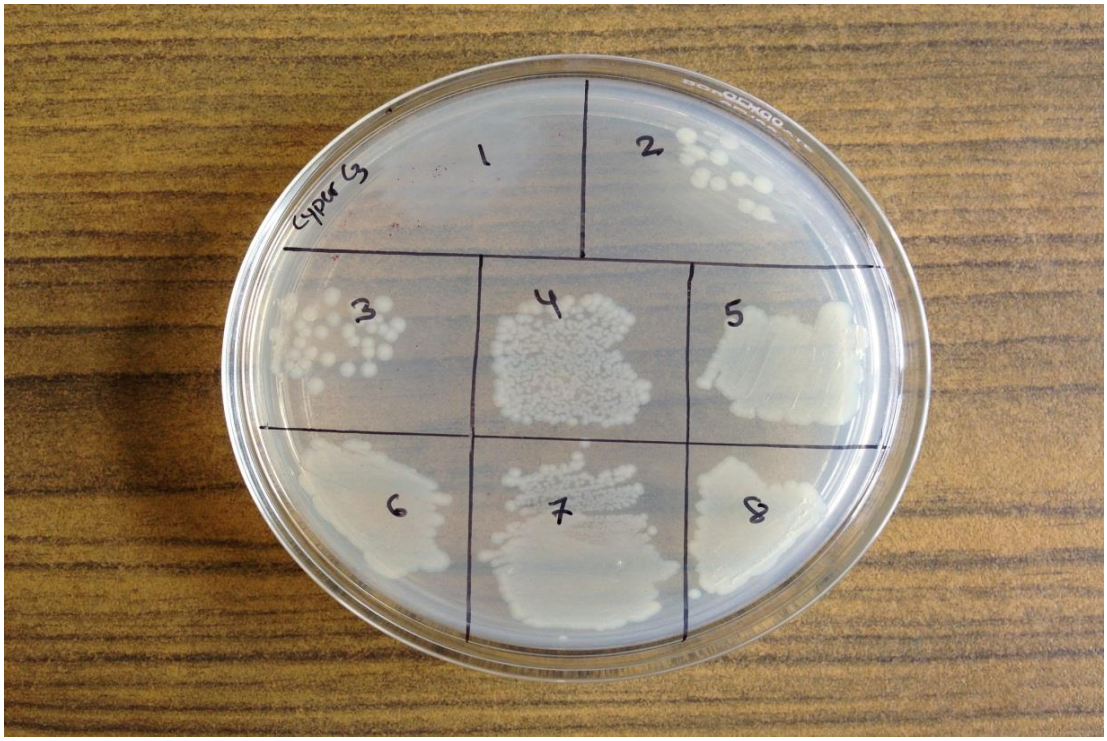

Fig: MBC of Cypermethrin 25% for biofilm producing *E. coli*; sample: C_{S3}; concentrations: 1, 2, 3, 4, 5,…: 25%, 12.5%, 6.25%, 3.13%, 1.56%,.....

Photograph 10

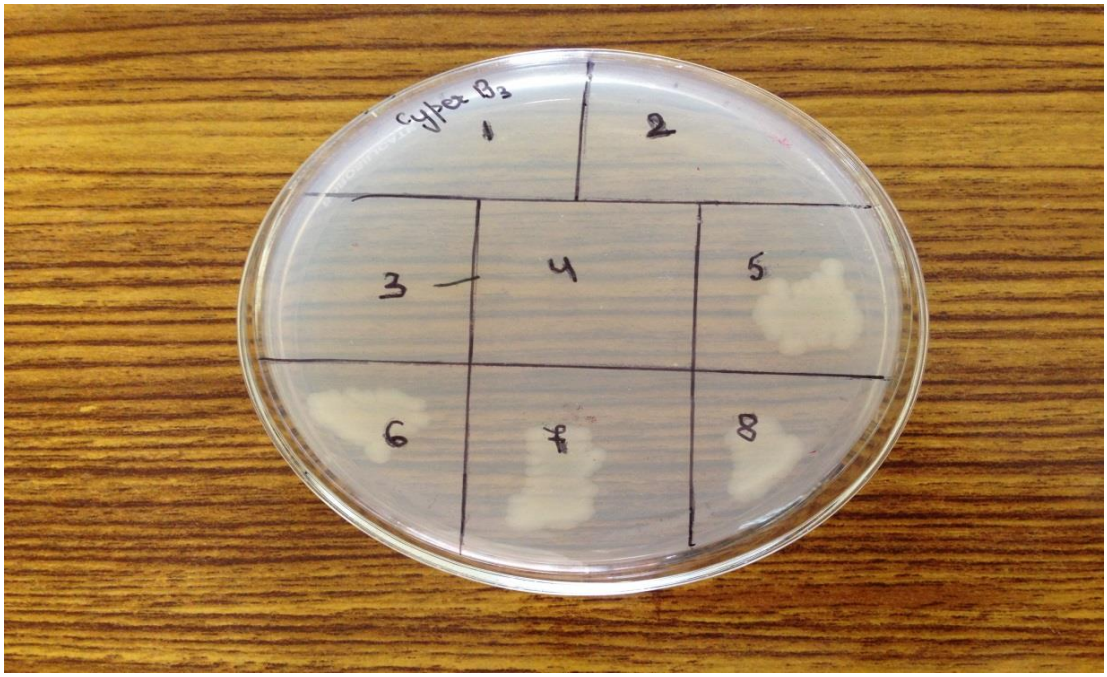

Fig: MBC of Cypermethrin 25% for biofilm non-producing *E. coli*; sample: $B_{S3;}$ concentrations: 1, 2, 3, 4, 5,… : 25%, 12.5%, 6.25%, 3.13%, 1.56%,…..

Photograph 11

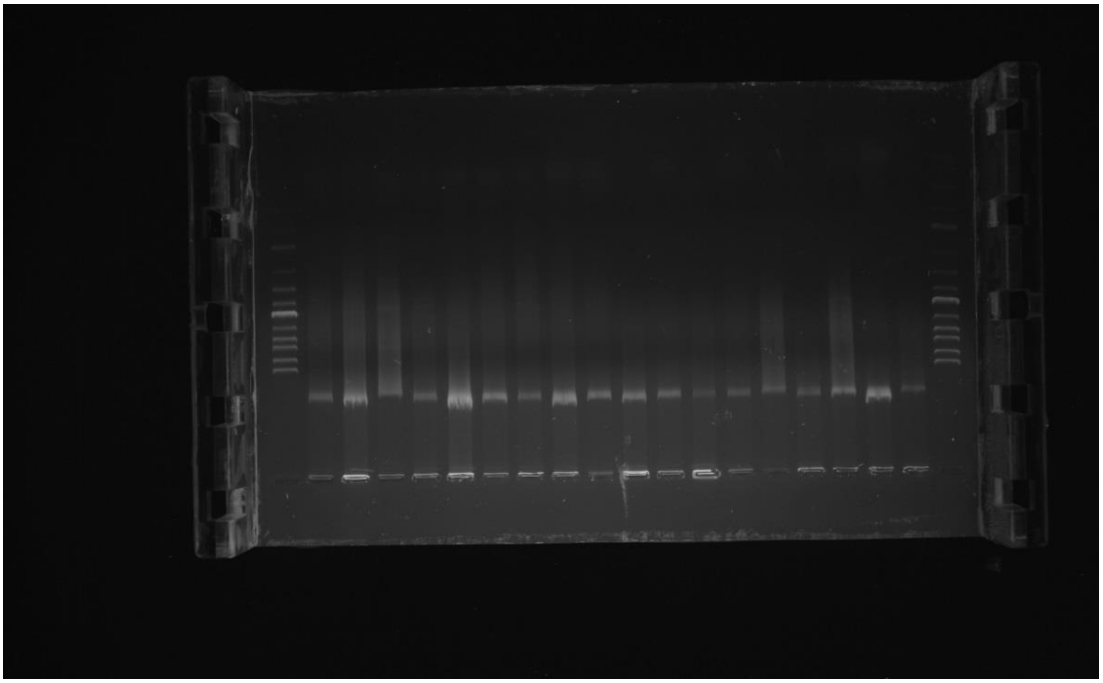

Fig: Plasmid profiling. Lanes (1,20): DNA ladder 10kb. Lanes (2-19): Samples- $C_{P1,}$ $Cu_{P1,}$ Cu_{S1}, $Cu_{S2,}$ $R_{K3,}$ $C_{K2,}$ $Cu_{K3,}$ $B_{S3,}$ $C_{P3,}$ $R_{S3,}$ $Cu_{P3,}$ $R_{P2,}$ $Cu_{K1,}$ $R_{K1,}$ $B_{P2,}$ $B_{K2,}$ $C_{S4,}$ R_{P1}. Lanes (2-19): plasmid band

Photograph 12

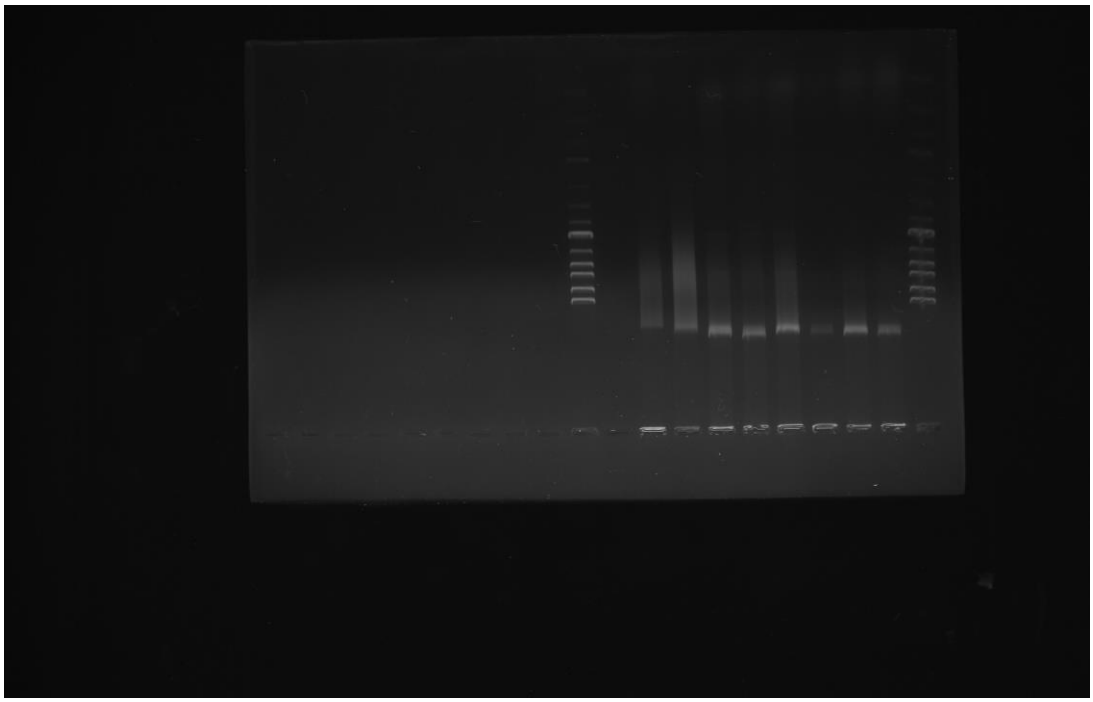

Fig: Plasmid profiling. Lanes (1, 11): DNA ladder 10kb. Lanes (2-9): Samples- T_{S3}, R_{P4}, R_{S1}, C_{P4}, C_{S3}, $C_{K1,}$ R_{S2}, and $R_{S4.}$ Lanes (2-9): Plasmid band. Lane 5, Lane 7: double plasmid bands at approx. 3kb and >10kb. Lane 6: triple plasmid bands at approx. 2.7kb, 3kb and >10kb. Lane 10: Control.

Photograph 13

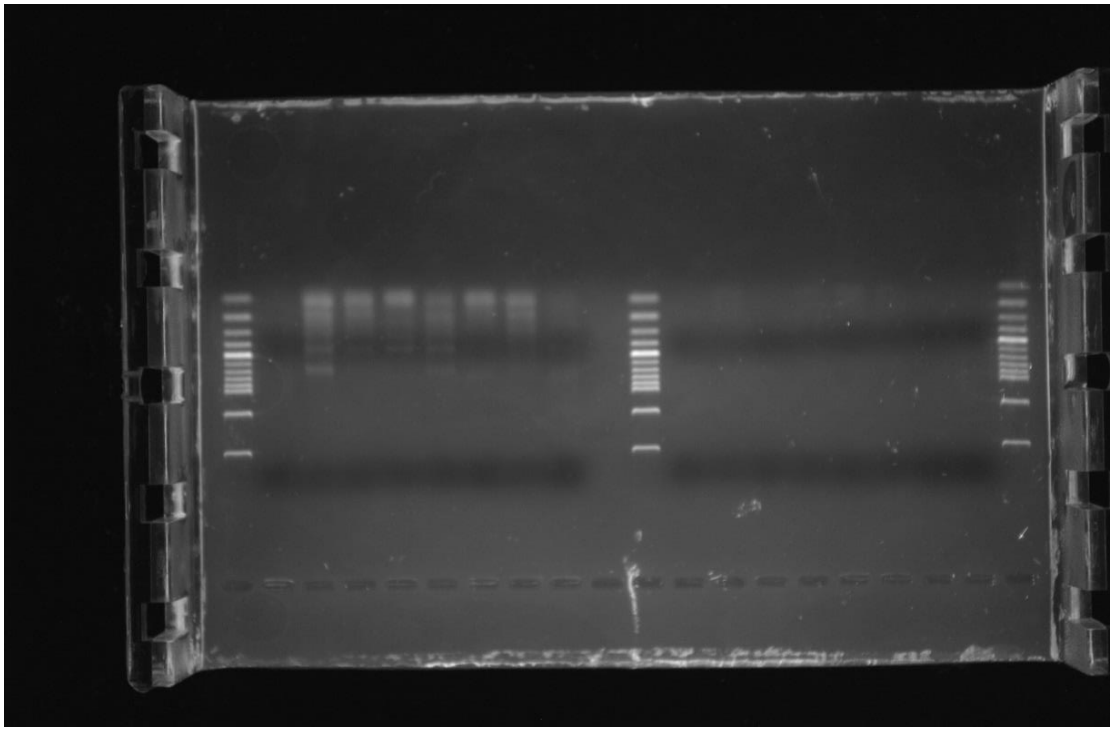

Fig: Agarose gel electrophoresis of PCR amplifications demonstrating presence of *stx* genes. Lanes (1, 10, 19): DNA ladder 3kb. Lanes (2-9): PCR amplified stx_2 gene. Lane 15, Lane 16, Lane 17, Lane 18: stx_1 bands (Samples: C_{K1}, C_{K2}, C_{P1}, C_{P3}). Lane 11: Control.