Virulence Profiling of *Enterobacter* Specie Isolated from Mass Gatherings of Pakistan

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بسم الله الرحمن الرحيم

DEDICATION

***To my loving mama, papa** and **brothers** for their endless support and love who always encouraged me to work harder in my field of interest and put my entire efforts to it.*

DECLERATION

I hereby declare that research work titled “Virulence Profiling of Enterobacter Specie Isolated from Mass Gatherings of Pakistan” is my own work. The work is novel and has not been presented elsewhere for assessment. Where material has been used from other sources it has been properly acknowledged/ referred.

Iqra Hoorain

CERTIFICATE

This dissertation, submitted by **Ms. Iqra Hoorain** to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the requirement for the degree of Master of Philosophy in Microbiology.

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Abbreviations

AMR	Antimicrobial Resistance
CDC	Centre for disease control and prevention
CRE	Carbapenem resistant Enterobacteriaceae
AMRCV-I	Crystal Violet-I Complex
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
ESBL	Extended-Spectrum B-Lactamase
etc.	Etcetera
Fig.	Figure
g	Grams
HGT	Horizontal Gene Transfer
ICU	Intensive care unit
hrs	Hours
i.e.,	That Is
MGs	Mass Gatherings
Min	Minute
MDRO	Multi Drug Resistant Organism
mL	millilitre
mm	millimetre
spp.	Species
UK	United Kingdom
US	United States
WHO	World Health Organization
α	Alpha
β	Beta
γ	Gamma
°C	Degree Celsius/Centigrade

%	Percentage

Acknowledgement

All Lord and glory to I love almighty Allah, the merciful and the most beneficent who's but no benevolence is everlasting, whose blessings are uncountable, who's being is eternal, and by his name we began this project. All the salutation and praises to be his beloved prophet and our true leader Hazrat Muhammad (SAW), who born for enlightening and guided in every aspect of life.

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Iqra Hoorain

Abstract

Mass gatherings (MGs) have been recognized as a major risk for disease outbreak by World Health Organization. In Pakistan, there is tradition of hosting mass gatherings, which include religious festivals, political, cultural, sports, food, and musical events. In MGs, due to proximity of people, poor hygiene, and cross contamination, it can be source of outbreaks especially from food and abiotic surfaces. The *Enterobacter* spp. are emerging opportunistic pathogens and are widespread in environment. The study aimed to determine the phenotypic analysis of virulence factors of *Enterobacte* spp. isolated from food and abiotic samples from different mass gatherings of Pakistan. A total of 52 food samples and 73 abiotic samples were collected from different mass gatherings. From these food samples, 83 Gram positive cocci and Gram-negative rods isolates were obtained. Meanwhile, 142 bacterial isolates from abiotic samples were purified. From these samples, total 117 Gram negative rods were isolated, and only 23 isolates were confirmed to be *Enterobacter* spp. after morpho-chemical characterization from both food and abiotic samples. A total of 50 *Enterobacter* spp. were subjected to different phenotypic virulence assays. Among these 50 isolates, 27 *Enterobacter* spp. were previously identified from food and abiotic samples from MGs. Among 23 newly identified *Enterobacter* species, 12 were isolated from food samples and 11 were isolated from abiotic samples. In case of food samples, 4 *Enterobacter* spp. isolates were identified from regular MGs and 8 *Enterobacter* spp. were identified from special MG events. Among 27 previously identified *Enterobacter* species, 17 isolates were from food samples and 10 isolates were from abiotic samples. None of the *Enterobacter* isolate had hypermucoviscosity. The 14% isolates from abiotic samples had beta hemolysin activity. A total 96% of isolates showed positive results for haemagglutination with blood group A. The Congo red assay showed 24% isolates were strong biofilm former and MTP assay showed 46% were strong biofilm former. Overall, 23/50 isolates exhibited strong biofilm formation according to MTP assay, out of these 12(52%) isolates we from special MG events and 11(48%) were from regular MG events. A strong association of hemagglutination activity (blood group A) with biofilm formation ability was observed in 100% *Enterobacter* isolates. Similarly, strong association of beta haemolysin activity with hemagglutination activity (blood group AB) was observed in 100% *Enterobacter* isolates. Thus, the overall results suggest that pathogenic *Enterobacter* isolates were prevalent in these mass gathering. On basis of this study, it is suggested that there is a need for training of food handlers and strict compliance to food biosafety regulations is need of time to prevent possibility of food borne outbreaks in MGs.

Chapter 1: Introduction

1.1 Mass Gatherings

According to the World Health Organization (WHO), Mass gatherings (MGs) are termed as "the presence of a large number of people at a particular location, for a particular time, and for a particular defined purpose" and the size limit lies within 1000 to more than 25000 individuals (WHO, 2015). Most MGs are recurrent and happens every year at same time and at the same location (*e.g.,* Hajj) or at different location (*e.g.,* sport games), though some events change every year for instance food festivals, at different time and location. Mass gatherings can be planned or spontaneous, which include religious, cultural, political, and social gatherings. Political rallies or funeral of well-known person are included in spontaneous mass gatherings. Due to lack of proper planning, it is very hard to manage spontaneous MG events (WHO, 2015). These gatherings globally are of different types; sport events (the Olympic Games, the FIFA World Cup, cricket world cup), social events (concerts and musical festivals), and religious ceremonies (*e.g.,* Hajj and Umrah), and political events (*e.g.,* rallies). Mass gatherings were also observed in Pakistan like religious events: Ashura, Urs at the Shrines, Eid-ul-Fitr and Eid-ul-Azha, cultural festivals, and food festivals. Figure 1.1 depicts the classification of MG events.

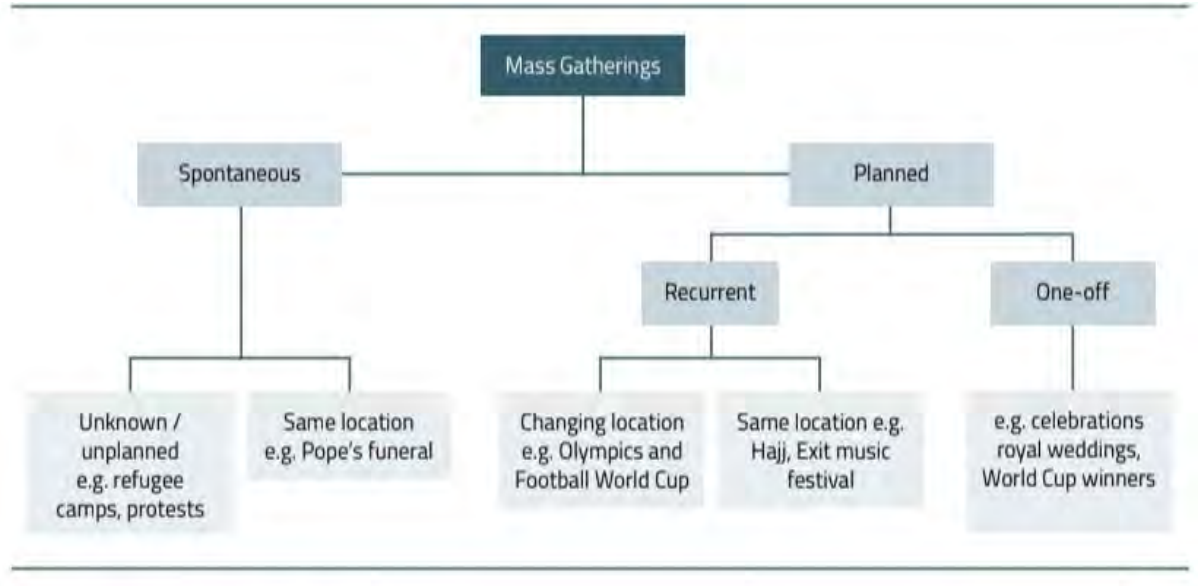

Figure 1.1 Classification of MG events adapted from WHO (2015)

During MGs, public health problems and outbreaks are mostly excepted risk, which are associated with population dynamics along with their behaviours, are emerging cause of concern for health community (Tabatabaei & Metanat, 2015). Even in Pakistan, there has been some reports of outbreaks at MGs. An outbreak was reported in 2010 at Urs of Baba Farid in

Pakpattan in which 500,000 people participated. 58% of 5918 people visited 15 healthcare facilities affected by communicable diseases, including 26% of gastrointestinal illnesses (Hassan *et al*., 2013).

1.2 Mass Gatherings and Infectious Diseases

MG, either planned or spontaneous, provides a gateway for transmission of pathogenic organisms due to the favourable environment and no proper distance between the large number of people increases the risk of transmission. The pathogenic microbial agents can be transmitted via direct and indirect routes for instance droplet, or direct contact with infected individuals during MG events (Memish *et al*., 2019; Zumla *et al.,* 2022). From epidemiological perspective, it can be major cause of transmission for infectious diseases as it was reported that less than 1% of infectious diseases were seen in 1996 Olympic Games at Atlanta (USA) and the 2000 Olympic Games in Sydney, Australia (Al-Tawfiq & Memish, 2012). Most of the previous outbreaks reported from mass gatherings are associated with the Hajj pilgrimage as large number of attendees from different countries perform hajj (Rashid *et al*., 2008). In 2023, approximately 2 million Muslims pilgrims from 180 different countries had participated in Hajj for specific time duration (Alandijany, 2023). Apart from highly coordinated and organized event, the authorities still prepared for risk for transmission of pathogenic microbes as it anticipated to be potentially high due to nature of Hajj rituals and overcrowding (Hoang & Gautret, 2018). In August 2017, foodborne outbreak was reported among Hajj pilgrims with ingestion of contaminated rice and kubah (Alaslani, 2018).

In mass gatherings, due to limited space, the food-preparation and storage areas had suboptimal conditions, insufficient food hygiene due to which food-borne outbreaks develop. Another important aspect of MGs is increased risk of transmission of communicable diseases (Yom-Tov *et al*., 2014). The transmission routes include respiratory, vector-borne, zoonotic, fomites and faecal oral route especially gastrointestinal disease (Al-Tawfiq & Memish, 2012). Multiple factors enhance the potential risk of transmission of pathogenic microorganism in MGs. These factors include overcrowding in limited space, lack of proper hygiene managements, restricted accessibility, lack of standard operating procedures (SOPs), sources of infection, strength of infectious agent and lack of knowledge among individual leading to disease outbreaks (Soomaroo & Murray, 2012). MGs pose a threat to public health, which is not restricted to hosting country but can spread worldwide for example COVID-19 (Ebrahim & Memish, 2020).

1.3 Mass Gathering and Foodborne Illness

Foodborne illnesses are a major global health concern. As in MGs, food is prepared for large quantity and during preparation, there are chances of food contamination due to improper food handling, raw material, storage, and insufficient hygienic practices. Upon ingestion of contaminated food, there is risk for foodborne disease outbreak (Augustin *et al*., 2020). In different countries food borne outbreaks make the major disease burden like the Integrated Disease Surveillance Project of India, reported that 60% of outbreaks are food poisoning outbreaks (Newman *et al.,* 2015). In Maharashtra (India), a religious mass gathering of 4000 individuals of all age groups 291 persons had food poisoning outbreak due to consumption of contaminated food for lunch. Their symptoms were diarrhoea, fever, chills, abdominal cramps, and vomiting (Bajaj & Dudeja, 2019). In 2019 another food borne outbreak occurred in planned MG event in Malaysia, consisted of 20,000 people including politicians, public members, and students from four public universities. The outbreak occurred due to consumption of the pre-packaged food. A total of 156 cases were reported with vomiting, abdominal pain, and diarrhoea, almost 49.1% (Rajakrishnan *et al.,* 2022). The food bone diseases in mass gathering causes the high burden on the local facilities.

1.3.1 Foodborne Diseases

Foodborne diseases are caused by ingestion of contaminated food by food borne pathogen (Bintsis, 2017). The Food borne diseases have been classified as; Food borne intoxications or food poisoning, and Food borne infections as shown in Figure 1.2 (DeWaal *et al.,* 2006).

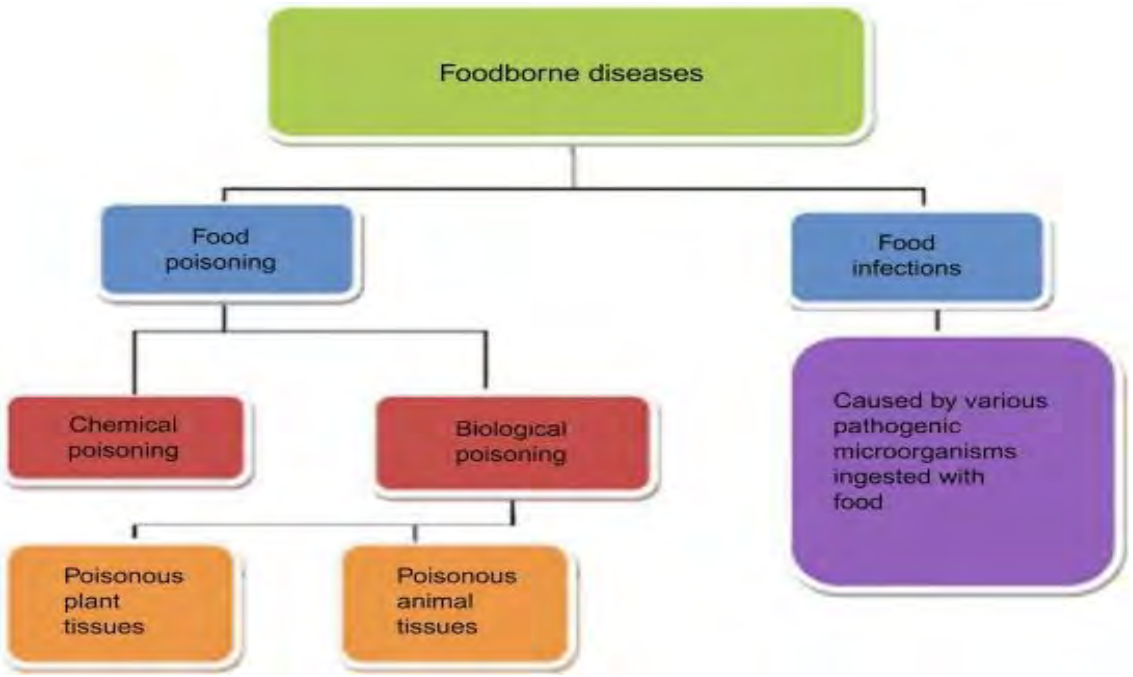

Figure 1.2 Classification of foodborne diseases adapted from Bari & Yeasmin, (2018)

Food consumption which contains poisonous compounds, bio toxicants (produced from tissue of plants and animals) and metabolic toxins (produced and released by microorganisms like bacteria, fungi, and algae) are key factors of foodborne intoxications (Sudershan *et al*., 2014). The food borne intoxications are categorized as:

- Bacterial intoxications (Shiga toxin by *Shigella)*
- Fungal intoxications (Aflatoxins by *Aspergillus flavus*)
- Plant intoxications (Lectins in beans)
- Chemical intoxication (Methyl mercury from industry)

Food borne infections are caused by the ingestion of contaminated food with pathogenic microorganisms. These microorganisms may be parasitic, viral, bacterial, or fungal (Gallo *et al.* 2020). The list of food borne infections due to bacteria includes cholera, salmonellosis, typhoid fever, shigellosis, yersiniosis, campylobacteriosis, *Escherichia coli* infection, *Vibrio parahaemolyticus*, and listeriosis. Viral foodborne infections include poliomyelitis virus, norwalk virus and hepatitis A. The fungal foodborne infections involve *Sporothrix* spp., *Candida* spp., and *Wangiella* spp., *etc*. (Akhtar *et al.* 2014).

1.3.2 Clinical Manifestations of Foodborne Disease

The main symptoms of foodborne diseases directly depend upon the raw material of food (Kushwaha *et al.,* 2008) or food contamination (Bintsis, 2017). The symptoms include abdominal pain, bloody or simple diarrhoea, vomiting, fever and chills. The disease symptoms

can last from a few days to months and can also become chronic diseases (Hoffmann & Scallan, 2017). Some bacteria like *C. botulinum* releases, toxins which can be damaging the nervous system with the symptoms of blurred vision, paralysis, headache, dizziness, numbness or tingling of the skin and weakness (Fein *et al.,* 1995). The One Health Concept focuses that the health of environment, animal, and human are interlinked, but criteria of food-borne infection convergence are undefined and complex (Lake & Barker, 2018).

1.3.3 Foodborne Illness and its Dynamics

The main reason of foodborne illness involves dynamic interaction of multiple agents which are:

1. Pathogenic agent (bacteria, viruses, and parasites)
2. Host (human and animal reservoir)
3. Environment (Hedberg, 2019)

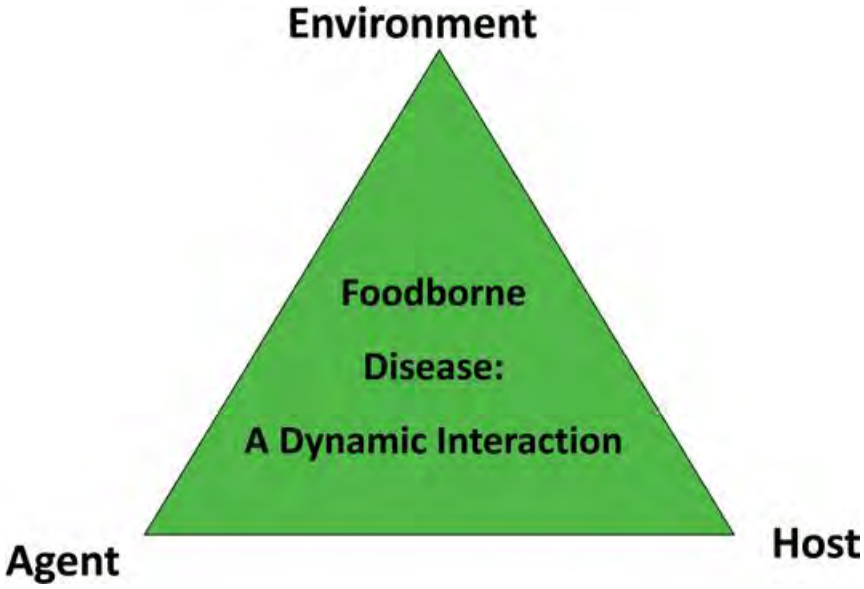

Figure 1.3 Epidemiological triad for foodborne illness adapted from Hedberg, (2019)

This relationship is referred as “Epidemiological triad”. It illustrates that there is need for the integrated information of various domains for comprehending foodborne illness and its origin. This knowledge suggests that there is dynamic nature of these components which indirectly affect the epidemiology of foodborne illness (Egan *et al.,* 2007; Hedberg, 2019).

Different food-borne diseases caused by biotic, even abiotic factors are also very important in causing the vomiting, nausea, abdominal cramps, bloody or water diarrhoea and gastroenteritis. *Campylobacter* spp., *Salmonella*, *S. aureus*, *Enterobacter* spp. *etc.* are some of the common

examples of causative agents involved in food-borne infections (Heeyoung Lee, 2021). The presence of coliform bacteria in food is of major importance alongside other foodborne pathogens, as their presence in food indicates the unhygienic conditions in the food processing environment (Martin *et al*., 2016).

1.4 *Enterobacter* Species

These are Gram-negative, facultative anaerobic bacteria and important opportunistic enteric pathogens belong to Proteobacteria phylum and Enterobacteriaceae family. Seven families belong to order Enterobacterales. It includes genus, *Escherichia, Yersinia, Klebsiella, Enterobacter, Salmonella, Shigella, Proteus, Serratia*, and *Citrobacter* (Patel & Patel, 2016).

Enterobacter spp. are emerging pathogens which have attained immense importance and recognition in recent years as are reported to be potentially pathogenic worldwide (Michidmaral Ganbold *et al*., 2023). *Enterobacter* spp. belongs to the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species*), which is a leading cause of nosocomial infections due to emergence of high resistance against antibiotics (Dinesh & Karthick, 2018). In 2016, European Centre for Disease Prevention and Control published data in which *Enterobacter* spp. was reported to cause 10% of pneumonias, 5% of urinary tract infections, and 8.5% of bloodstream infections with 32% isolates being resistant to third generation cephalosporins isolated from intensive care units of hospitals in Europe (European Centre for Disease Prevention and Control, 2018). But now, it is also seen as an emerging food-borne pathogen as various studies document it (Yong *et al.,* 2018). These coliform pathogens can spread through improper food handling practices, inadequate sanitation measures, or through contaminated food or water. Vulnerable patients, including those with compromised immune systems or undergoing medical procedures, are at particular risk of nosocomial foodborne infections (Zahra, 2023).

The *Enterobacter* spp. lies among the global priority and emerging opportunistic pathogen, possessing different antimicrobial resistance patterns and multiple virulence properties. According to recent report, *Enterobacter* spp. is the second-highest prevalent microorganism among five different food categories from animal origin (Edris *et al.,* 2023). This microorganism has the potential to form biofilm, some *Enterobacter* spp. are very strong biofilm formers in food environment (Edris *et al.,* 2023). As biofilm forming pathogen if ingested by human, there is probability that those biofilm formers might adhere to epithelial

cells and cause infection, which can be a major health hazard for human population (Oluwole, 2022). The other major virulence factors of this bacteria include exotoxins, endotoxins, adhesins, motility, two-component system, quorum sensing, and iron acquisition system (Cunliffe, 2008).

In Pakistan, annually different types of MG events take place and food is prepared in large quantities. But due to limited space it is observed that handling of food is improper, cleaning and sanitation facilities are poor, and highly unhygienic practices of delivering food to consumers increase the risk of faecal contamination and cross contamination, it might be reason for foodborne illness. Until now, no specific data has been published so far on isolation and virulence profiling trend of *Enterobacter* spp. from MGs of Pakistan.

Aims and Objectives

Aim:

The aim of the study was to determine the virulence determinants of *Enterobacter* species isolated from different mass gatherings of Pakistan.

Research Objectives:

The objectives of this study include:

- Survey of the mass gatherings to estimate the compliance of food safety regulations and practices.
- Isolation of *Enterobacter* species from food as well as abiotic samples using standard microbial methods and identification through biochemical characterization.
- Virulence profiling of *Enterobacter spp.* by using different phenotypic virulence assays.

Chapter 2: Literature Review

2.1 Foodborne Illnesses

Several reports regarding foodborne illness had been reported. Some of the foodborne pathogens associated with outbreaks were mentioned in Figure 2.1. *Escherichia coli* O157:H7 was first recognized as a human pathogen in 1982. It caused bloody diarrhoea due to consumption of inadequate cooked hamburgers and showed symptoms of haemolytic uremic syndrome among children (Riley *et al.,* 1983). Global foodborne outbreaks involving food products like raw milk, fresh cheese, curd, and unpasteurized fruit juices made of fruits like apples are exposed to bovine manure and seeds get contaminated. From USA exported spinach have been reported in publications to be involved in food borne infections (Espié *et al.,* 2006; Doyle & Erickson, 2008; Heaton & Jones, 2008; Vojdani *et al.,* 2008). An outbreak almost 6955 cases of bovine spongiform encephalopathy was reported from UK due to the consumption of meat contaminated with Transmissible Spongiform Encephalopathy (TSE) and was considered as an emerging public health threat (Grist, 2007; Ducrot *et al.,* 2008). Meat processing methods like the dry rendering of animal carcasses was a leading cause of tissue contamination by TSE effecting the food chain (Cooper & Bird, 2002; Caramelli *et al.,* 2006; Doherr, 2006).

According to the Centre for Disease Control and Prevention (CDC), each year more than 9 million incidents of foodborne illness are reported, with over 55,000 hospitalizations and 1,351 deaths, that occur due to contaminated food consumption in the USA (Scallan *et al.,* 2011). According to CDC from 1998-2008, total of 13,405 food borne outbreaks, 273,120 cases of illness, 9109 hospitalizations, and 200 deaths were reported. The causative agent were bacteria (45%), viruses (45%), and remaining were due to parasites, chemical and toxic agents (Gould *et al.,* 2013). The major causative factors are lack of proper hygiene practices, lack of handwashing practice, no proper use of gloves, head nets by food handlers, time temperature misuse and improper food storage.

2.2 Microbial Transmission in Food from Different Sources

Microorganisms get access to food by both natural sources and external sources, from the time of production till the time of consumption. Natural sources of microbial contamination are surface of fruits and vegetables, spices, grains, and raw meat. Also, natural microflora of source depends on factors like host, type of ecological level, geographical location, and environmental conditions. The food products of animal origin such as meat and milk have the highest rate of pathogen's prevalence, chemical contaminants, adulterants, and natural toxins. According to

study in 2003, high consumption of animal source food products leads to increase death rate, because use of antibiotics in livestock animals, due to excessive use there is increase in antimicrobial-resistant microorganism leading to illness in humans (Barber *et al*., 2003).

Apart from this, external source includes different types of microbes from air, soil, water, food ingredients and equipment. This interaction of microorganism with food depends on sanitation condition maintenance, hygienic condition of the food production environment, handling practices of food and health condition of workers (Erkmen & Faruk Bozoglu, 2016; Dodd *et al.*, 2017). Table 2.1, Figure 2.1, and Table 2.2 provides a detailed description of types of microorganisms and their source.

Along with that, contamination of food products may also occur at any level of the food chain or at any division of food production. Therefore, proper knowledge regarding the food safety hygienic condition practices, can reduce the risk of contamination in food (Mudey *et al.,* 2010).

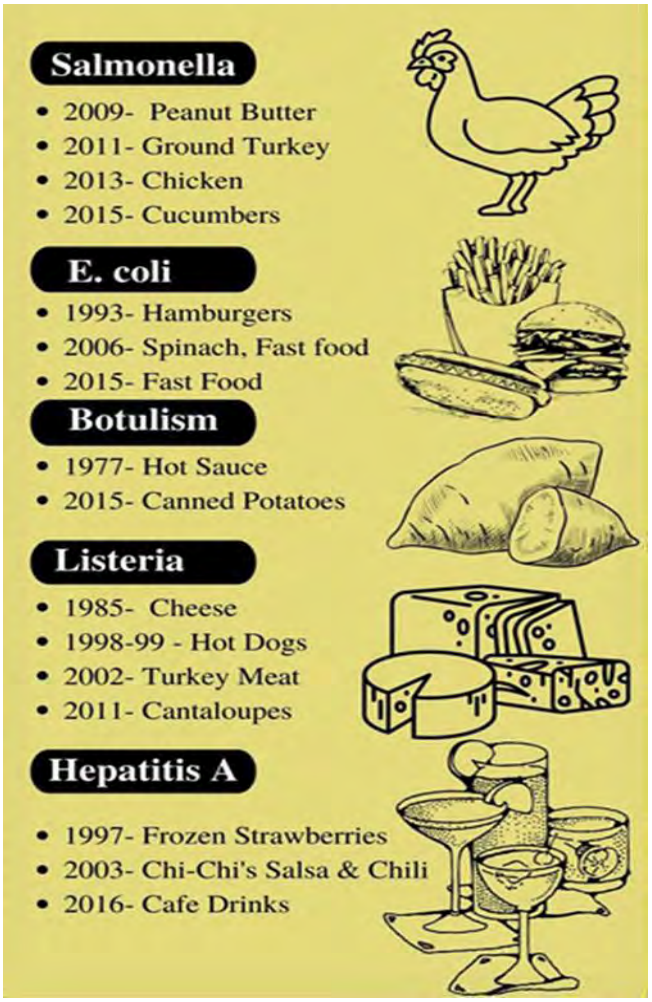

Figure 2.1 Foodborne pathogens with outbreaks adapted from Shakeel *et al.,* (2023)

Table 2.1: External and internal source of microbial diversity to food

Predominant Source		**Type of microorganisms**	
Natural or internal sources		**Natural microflora**	**Pathogenic Microorganism**
Plants	Vegetables and fruits	*Pseudomonas, Alcaligenes, Micrococcus*, *Erwinia*, *Bacillus*, *Clostridium, Enterobacter*	*Salmonella*, *Escherichia coli*, *Campylobacter*, *Shigella*, *Cyclospora*, *Giardia*
Animals	Birds		*Salmonella, Escherichia coli, Campylobacter jejuni*, *Yersinia enterocolitica*, *Listeria monocytogenes, Staphylococcus aureus*, *Micrococcus* spp., *Propionibacterium* spp., *Campylobacter*, *Corynebacterium* spp.,
	Fish and Shellfish		*Staphylococcus aureus*, *Micrococcus* spp., *Propionibacterium* spp., *Campylobacter*, *Corynebacterium* spp., *Vibrio parahaemolyticus*, *Vib. vulnificus*, and *Vib. cholerae*

	Meat		*Bacteroidetes, Prevotella,* and *Bacteroides; the Firmicutes Faecalibacterium, Ruminococcus, Roseburia,* and *Clostridium; E. coli, Salmonella, Bacillus, Corynebacterium, Paenibacillus, Micrococcus,* and the moulds *Alternaria, Cladosporium,* and *Penicillium* etc.
External Sources			
Air			Spores of *Bacillus* spp., *Clostridium* spp., *Micrococcus* spp. and *Sarcina* spp. and viruses (including bacteriophages)
Soil	Used in agriculture and for raising animals	*Enterobacter*, *Pseudomonas*, *Proteus*, *Micrococcus*, *Enterococcus*, *Bacillus*, and *Clostridium*	Enteric pathogens
Sewage and Manure	Used as fertilizer		Enteric pathogens
Water		*Pseudomonas*, *Alcaligenes*, and *Flavobacterium*.	Coliforms and enteric pathogens

Table 2.2: Source of transmission of microbes from inanimate and human sources to food

Category		**Microbial Diversity**
Inanimate source		
Food ingredients		Rich in moulds and bacterial spores
Equipment	harvesting, transporting, slaughtering, processing, and storing foods	*Salmonella*, *Listeria*, *Escherichia*, *Enterococcus*, *Micrococcus*, *Pseudomonas*, *Lactobacillus*, *Leuconostoc*, *Clostridium*, *Bacillus* spp., and yeasts and moulds
Human Source		
Human	Production, Consumption, Handling	*Staphylococcus aureus*, *Salmonella* serovars, *Shigella* spp., pathogenic *E. coli*, norovirus, and hepatitis A etc,

2.3 Foodborne Disease Burden

Approximately 76 million people suffer from food-borne diseases and 5000 deaths occur in USA each year (Newell *et al.,* 2010). The disease burden is high in low middle-income countries due to high poverty level and insufficient health facility, hence effecting health of more people at the global scale (Grace, 2015; Shakeel *et al.,* 2023). With the rise in global trading between international markets and the food chains complexity, contamination rate of food is rising as infected food products were transported across the globe. Climate change, international trade, urbanization, migration, and tourism had increased the spread of pathogens and contaminants in food (Bhaskar, 2017). In USA, 30 foodborne diseases cause 48.7 million cases in a year due to food contamination (Newman *et al.* 2015).

2.4 Prevalence of Foodborne Diseases in Pakistan

Pakistan is affected by foodborne pathogens via ingestion of contaminated water or meat, and improper cooked foods (Ishaq *et al.,* 2021). The most common agents of infectious foodborne disease in Pakistan include Norovirus (44%), *Campylobacter* (7%), *Clostridium* (8%), *Salmonella* (8%), *Listeria* (15%), and Toxoplasma (18%). Echinococcus granuloses from meat is also major concern in Pakistan's southern agricultural areas due to presence of cystic echinococcosis in 9% of animals, including buffaloes 12%, cows 9%, sheep 10%, and goats 5.1% (Khan *et al.,* 2021). *Campylobacter* was extensively reported from various food items like vegetables and fruit salads (40.9%), raw chicken (48%), raw mutton (5.1%), raw beef (10.9%), cheese (11%), sandwiches (32%), and milk (10.2%) (Ishaq *et al.,* 2021; Modesto dos Santos & Modesto Sugai, 2022). A study reported the prevalence of food borne pathogens in street food (samosa, roll, chaat, ban kabab, and pakora), which included *Escherichia coli, Pseudomonas, Salmonella* and *Enterobacter.* The study showed that street food was contaminated due to mishandling during the preparation, storage, and serving of food items, the personnel hygienic and sanitation condition of vendors, and faecal contamination due to environmental sanitation condition (Alamgir *et al.,* 2023). To prevent and monitor these diseases, different effective methods like a good hygienic environment, food pasteurization, and execution of standard hazard analysis critical control point (HACCP) protocols should be used (Newell *et al.,* 2010).

2.5 Factors affecting the Foodborne Illness

There can be multiple factors responsible for foodborne illness like survival of microorganism in food, time, temperature, cooking practices, hygienic conditions, and cross contamination of food. The findings from year 1991 to 2021 revealed that outbreaks of foodborne illness were associated with "Temperature abuse" (70.3%), followed by contamination (48.5%) and "cross-contamination" (39.6%) (Dein Warmate & Onarinde, 2023). Due to poor food handling practice around 600 million foodborne illnesses and 420000 deaths occur each year (WHO, 2015).

2.5.1 Behaviour of Microorganisms in Food

The impact of microbial density in general environment including food over time is depicted by one of its three behavioural modes, which includes growth, survival, and death (Velusamy *et al*., 2010).

2.5.1.1 Growth Behaviour of Microorganisms in Food

Microorganism growth in food begins with cross contamination. Due to this, different types of mesophiles, psychrophiles and thermophiles microorganisms could be present in food (Chen *et al.,* 2017). Under favourable conditions, the microbes start to divide exponentially, which depends on variable conditions like carbon or nitrogen source, water, pH, and temperature (Ezraty *et al.,* 2017). Microbial population varies during spoilage and storage of food (Pitt, 2014). Food spoilage results in food poisoning, which is an outcome of microbial growth. These microbial growth activities can result in alteration in the food pH, odours, flavours, colour and formation of gas or slime layer (Skovgaard, 2010). Different toxins are also produced in food for example staphylococcal enterotoxins (Cao *et al.,* 2012). The comprehension to this growth kinetics enables the detection of specific type of microbial community present in different types of food (Fang *et al.,* 2016).

2.5.1.2 Survival Behaviour of Microorganism in Food

In survival stage of microorganism neither growth nor death of microorganism takes place, hence constant microbial density. Different practices at this level can prevent the microbial contamination and spoilage of food like use of a bacteriostatic agent (*i.e.,* growth inhibitor) to treat microbial growth. Factors like stress adaptation, dormancy, the viable-but-nonculturable state (VBNC) and persistence play a significant role in survival of microorganisms (Begley & Hill, 2015).

I. Stress Adaptation

Stress adaptation involves the alteration of gene expression upon unfavourable condition in surrounding. This survival strategy alters the tolerance of pathogenic bacteria, increases the chances of infecting host by altering the virulence characteristic of pathogenic microbe. (Wesche *et al.,* 2009). For example, studies revealed that virulence characteristics of *Salmonella* are linked with stress (Su *et al.,* 2008). Such type of bacterial response compromises the safety of food in a way like mild treatments during food production and processing could be escaped by such foodborne pathogenic microorganisms.

II. Persistence

Persistence is the ability of bacteria to survive for a longer period of time in a specific habitat due to colonization and biofilm formation (Kempf & Bremer, 1998). This causes repeated food contamination and spoilage, pose high risk for foodborne diseases (Fox *et al.,* 2011). For example, a study reported that *Salmonella* is more persistent due to its biofilm formation capability (Møretrø, 2003).

III. Dormancy

Dormancy refers to spore formation. Many species like *Clostridium* and *Bacillus* species undergo process of dormancy under unfavourable conditions (Nerandzic & Donskey, 2013). If food is stored with such pathogenic spore forming microbes, then when the favourable conditions are restored, they start to produce toxin, causing a potential risk for food poisoning (Bull *et al.,* 2008).

IV. Viable-But-Non-culturable State

VBNC state is a state when bacteria may not be able to grow on conventional media/culture but remains viable. This condition is very harmful as they go undetectable and pose a threat to public (Nicolò *et al.,* 2011). According to a report, 67 out of 85 species of bacteria which are pathogenic can enter into VBNC state (Zhao *et al.,* 2017). The bacteria maintained their virulence property and affect the cell when they revive back to normal state (Dinu & Bach, 2013). A study reported that enteropathogenic *E. coli* after VBNC state retain their entero-pathogenicity (Pienaar *et al.,* 2016). But some pathogens can become non-pathogenic like VBNC *Salmonella Typhimurium* can lost its virulence property and could not infect mice *in vitro* (Habimana *et al.,* 2014).

2.5.2 Cross Contamination

Cross contamination majorly contributes to foodborne illness (BRYAN, 1988). According to CDC, 12% of all outbreaks were linked to cross-contamination and was common contributing factor of foodborne illness (Dewey-Mattia *et al.,* 2018). The contamination in food products can occur on any level of the food chain or at any production level of food. The cross contamination of the food depends on the hygienic condition of the environment for food production and the health condition of workers (Mudey *et al.,* 2010). There is popular "five second rule" which states that the dropped food material on the floor for less than five seconds is “safe to eat”, because microorganism need time to transfer. However, researchers from Clemson University found that if food is dropped for longer duration (5, 30 and 60 sec) increased transfer rate of *Salmonella Typhimurium* from wood, tile, and bread occurs (Berto, 2007). There was another research conducted at Aston University (UK) in 2014, which showed that contact time predominantly affected transfer of both *S. aureus* and *E. coli* from contaminated surface like carpet and tile to food items like pasta, biscuit, toast and a sticky sweet (Miranda & Schaffner, 2016).

2.6 Inanimate Environmental Source and Microorganism

Inanimate surfaces are secondary reservoirs of microorganisms because there is transmission of microorganism from animated source to inanimate source present in the environment (Kramer & Assadian, 2014). If the conditions are favourable, these potential pathogenic microorganisms multiply and survive in the environment posing a risk of serious infection (Wißmann *et al.,* 2021). The main factor involved in transmission of pathogenic microorganisms are as follows:

1. Persistence of microorganisms on inanimate surfaces
2. Transmission of pathogens from inanimate surfaces to the host

The inanimate object or surfaces can be the major source of contamination as pathogenic microorganism may persistent upon them. The scientific findings depict different microorganism have different range of survival in the environment specifically on different abiotic surfaces (Table 2.3)

Table 2.3 Survival of different pathogenic microorganism on different surfaces (Wißmann *et al.,* 2021)

Pathogen	**Material**	**Survival Range**
Acinetobacter baumannii, Acinetobacter calcoaceticus, Acinetobacter radioresistens, Acinetobacter spp.	Stainless steel	6 days–>6 weeks
Campylobacter jejuni	Stainless steel and Formica	30 min–7 h
	Aluminium and Ceramics	15 min–7 h
Escherichia coli	Stainless steel	14–>60 days
	Plastic	24 h–>300 days
	Glass	1–_14 days
	Cloths	4 h–>8 weeks
Enterobacter spp.	Cloths	24–>48 h
Salmonella spp.	Plastics	>72 h–>300 days
	Stainless steel	>30 days
Klebsiella pneumonia	Cloth	<1 h–4 weeks
	Plastic	9–32 days
	Stainless steel	3–6 weeks
Neisseria gonorrhoeae	Plastics	>24 h
Proteus mirabilis	Cloths	4 h–9 days
	Plastics	<1–26 days
Pseudomonas aeruginosa	Stainless steel	5 days
	Plastics	9 h–10 days
Serratia marcensens	Plastics	1–10 days
	Glass	>7 h–11 days
Shigella dysenteria	Plastic	1.5 h

2.6.1 Transmission of Pathogens from Inanimate Surfaces to Host

The transmission of pathogen to host involves direct transmission and indirect transmission, that is cross contamination of food from human, equipment, or other food material. In direct transmission, contaminated surfaces serve as a vector for transfer of pathogens to host (Kramer *et al.,* 2006). Surface to hand transmission is a potential source of harmful bacteria and viral infection (Kramer *et al.,* 2006). On other hand, cross contamination can be from surface to food, hands to food, and food to surface (Jensen *et al.,* 2013). This transmission of pathogen from one source to other depends on factors like surface type, contact time, inoculum size, contact pressure, friction, and surface moisture (Merry *et al.,* 2001; Sattar *et al.,* 2001).

Most hospital environment are studied for transmission of pathogen, the main route of transmission of pathogenic microorganism from hospital environment is described in Figure 2.2. Different studies reported that *E. coli, Salmonella,* and *S. aureus* spp. were 100 % transmitted between fomites (Kramer *et al.,* 2006). Studies showed that hand hygiene was less frequent practice among healthcare workers after dealing with patient and surrounding environment (Randle *et al.,* 2010).

Figure 2.2 Transmission routes for nosocomial pathogens adapted from Kramer & Assadian, (2014)

2.6.2 Persistence of Microbes on Inanimate Surfaces

Various household, kitchen and other surface types have presence of potentially pathogenic microorganisms and cross contamination from these surfaces to food can pose a threat to infection. These surfaces include stainless steel, glass, wood, ceramic tile, carpet, and plastic (Kusumaningrum *et al.,* 2004; Dawson *et al.,* 2006; Lankford *et al.,* 2006; Jensen *et al.,* 2013). Generally, household kitchen items made of stainless steel are preferred because of their ability

to resist corrosion and chemical degradation, high mechanical strength, and easy cleaning for user (Wilks *et al.,* 2006). However, it was reported that stainless steel had high tendency of bacterial transfer than other surfaces (Wilks *et al.,* 2005). Different comparative studies had been done over hygienic properties of wood cutting boards and plastic cutting boards in the past (WELKER *et al.,* 1997). The United States Department of Agriculture recommends that there must be one cutting board specific for cutting each food item *i-e.,* one cutting board for bread and a separate cutting board for raw meat. Another abiotic surface *i.e.,* carpet seen in major houses is a favourable site of contamination (Rice *et al.,* 2003).

Factors affecting the environmental persistence of microbes (Tang, 2009) were as following.

- Relative Humidity (RH):

Survival of Gram-negative bacteria like *Pseudomonas* spp., *Enterobacter* spp. and *Klebsiella* spp. on inanimate objects was enhanced at higher relative humidity and low temperature.

- Temperature:

For bacterial survival, there are different temperature in which bacteria persist in different habitat, but it was reported that temperature >24℃ decreases the survival of airborne bacteria. Mostly the pathogens grow at temperature range from 20 °C to 45 °C and are mesophilic. Some of the pathogenic agents are psychrotrophs, which can grow under temperature lower than 10℃ these includes *Listeria monocytogenes*, and *Yersinia enterocolitica* (Bennett *et al.,* 2013; Bujňáková *et al.,* 2022).

- Biofilm:

Biofilm formation is a complex and dynamic process depending on the medium, matrix, cells intrinsic properties, cell metabolism, signalling molecules and genetic conditions (Renner & Weibel, 2011; Sadekuzzaman *et al.,* 2015). The main feature of biofilm includes protection of microorganisms from extreme pH, extreme temperature, ultraviolet radiation, high salinity, high pressure, antibiotics, and malnutrition (Yin *et al.,* 2019). The U.S. National Institutes of Health reported that 80% of bacterial infections are because of biofilm formation by microorganism (Worthington *et al.,* 2012).

2.7 Foodborne Pathogens

The term 'foodborne pathogens' is defined as pathogenic agents or biological agents responsible for causing foodborne illness or infections (Madilo *et al.,* 2023). The most common cause of foodborne illness are bacteria. Recent data indicated *Aeromonas hydrophila, Anthrax, Bacillus cereus, Campylobacter jejuni, Clostridium, E. coli, Enterobacter, Listeria monocytogenes, Salmonella, Shigella,* and *Staphylococcus* as major foodborne pathogenic bacteria (Dein Warmate & Onarinde, 2023). Also, spore forming, and heat-resistant pathogenic bacteria include *Clostridium botulinum, Bacillus cereus* and *Bacillus subtilis* infections (Stenfors Arnesen *et al.,* 2008). Even some bacteria like *Staphylococcus aureus* and *Clostridium botulinum* produces heat-resistant toxins and cause illness (Scallan *et al.,* 2011). Botulism is a rare but deadly illness causing Botulinum neurotoxins (Munir *et al.,* 2023). Food-borne diseases can be fatal as these depend on pathogenic microbial contamination and toxins production (Fung, 2009).

2.8 Genus *Enterobacter*

The genus includes Gram-negative bacilli which are motile and 2mm in length, possessing peritrichous flagella (Davin-Regli *et al.,* 2019). Almost 22 species have been reported in this genus which are: *E. aerogenes, E. amnigenus, E. arachidis, E. asburiae, E. carcinogenus, E. cloacae, E. cowanii, E. dissolvans, E. gergoviae, E. helveticus, E. hormaechei, E. kobei, E. ludwigii, E. mori, E. nimipressuralis, E. oryzae, E. pulveris, E. pyrinus, E. radicincitans, E. soli, E. taylorae,* and *E. turicensis* (Parte *et al.,* 2020). Among these species, *Enterobacter cloacae* complex is a group of seven different species: *E. cloacae, E. asburiae, E. hormaechei, E. kobei, E. ludwigii, E. mori,* and *E. nimipressuralis.* Recently identified species, includes *Enterobacter roggenkampii, Enterobacter bugandensis, and Enterobacter chengduensis* are clustered with the species of ECC (Doijad *et al.,* 2016; Sutton *et al.,* 2018; Wu *et al.,* 2019). Among them, *E. cloacae* and *E. hormaechei* are the most frequently reported in clinical infections and in foodborne outbreaks (Davin-Regli & Pagès, 2015; Davin-Regli *et al.,* 2019; Edris et al., 2023). The *E. cloacae* shares 60% genomic similarity with the other six members of the group (Hoffmann & Roggenkamp, 2003).

The genus Enterobacter is widely present in a variety of environmental habitats such as soil, water, the faeces of humans and animals, plant materials, insects, and dairy products and are endophytic or considered phytopathogens for various plant species (Singh *et al.,* 2018). These are commensal microorganism in healthy human and animal gut but are also opportunistic

pathogens. In healthy gut, the microbiota is balanced hence no infection, but any imbalance can trigger inflammatory response. Only certain subspecies/species had been linked with hospital acquired infections and outbreaks (Bertrand, 2003). *Enterobacter aerogenes* and *E. cloacae,* are reported frequently in nosocomial infections, *i.e.,* in intensive care unit (ICU) and in immunocompromised patients (Anastay *et al.,* 2013). *Enterobacter* spp. had been found in; respiratory tract (11.1%), surgical wound (10.3%), urinary tract (6.1%), and blood (5.3%). Studies showed that immunocompromised patients were found to be more prone to infection caused by *Enterobacter* spp. (Patel & Patel, 2016). *Enterobacter cloacae*, *Cronobacter sakazakii* (known as *Enterobacter sakazakii*) and *Enterobacter aerogenes* (known as *Klebsiella aerogenes*) were reported to cause diarrhoea in children and adults due to use of contaminated water for cooking and drinking (Farone *et al.,* 2011).

2.9 *Enterobacter* species in Food

Enterobacter spp. had been reported in a wide range of food items which include fruits, vegetables, tea, spice, herbs, legumes, animal feed, fish, meat (mutton or beef), eggs, powdered infant formula, dairy products, nuts, flour, grains and seeds, chocolate, pasta, water, and beverages. *Enterobacter cloacae* as pathogen can be identified in contaminated raw milk, cheese, yogurt, and other dairy produce (Oonaka *et al.,* 2010). Studies showed that *Enterobacter* spp. can become inactive during pasteurization, but it had been also found in pasteurized milk and cream, which might be because of postprocess contamination (Boor *et al.,* 2017). In any production facilities, a huge number of contaminants can enter via different routes for instance, through human and vehicular carriages, or through water leaks or as particles in the atmosphere (El-Zamkan & Mohamed, 2018). Reports have shown that *Cronobacter, E. pulveris,* and *E. helveticus*, have ability of high resistance and can persist in dry processing environments (Iversen, 2014).

A study from Pakistan reported highest occurrence of *Enterobacter* spp. in ready to eat food items like chaat samples (60%), Bun kabab and roll (50%), samosa (40%) and pakora (30%) (Alamgir *et al.,* 2023). This may be due to multiple factors as *Enterobacter* spp. can be isolated from surface water, drinking water, and soils. The study showed that the pathogen was also isolated from different sources like water, food, plants, soils, health care equipment, like probes, catheters *etc* and hospital environments (Richard, 1989). An outbreak of *Enterobacter* spp. was reported in a hospital due to the contamination of packed food stored in a refrigerator (Dugleux *et al.,* 1991).

2.10 Virulence and Pathogenicity of *Enterobacter* species

Pathogenicity is known as the ability of a microorganism to cause disease in a host (Casadevall & Pirofski, 2009). In the class Enterobacterales, almost all genera possess multiple virulence and pathogenicity factors which are involved in causing severe health issues (Amaretti *et al.,* 2020). These group of bacteria involve multiple strategies to enhance invasiveness and supress host defenses to cause infection. They have different virulence factors, which perform similar functions with minute alteration (Folgori *et al.,* 2021). The important virulence factors are adhesins, intimin, fimbriae, capsules, siderophores, (*e.g.,* enterobactin, aerobactin, yersiniabactin), protectines, allantoin metabolism (Lee *et al.*, 2017; Martin & Bachman, 2018), cell invasion (responsible for mucoid phenotype and invasiveness), heme/hemoglobin transport protein and receptor, and outer membrane porin A. The cellulose structure production, fimbriae and curli allow significant role in biofilm infections (Zogaj *et al.,* 2003; Tursi & Tükel, 2018).

These microorganisms have the ability to obtain mobile genetic elements/ transposons instantly from other bacteria that carry virulence and resistance genes. This possible exchange of genetic material via horizontal gene transfer implies a major global public health concern. Various studies have reported that strains with high resistance capability are more likely to exchange genetic material (Amaretti *et al.,* 2020).

2.10.1 Virulence factors of *Enterobacter* species

The microbial features, which are potent for exhibiting virulence falls in multiple categories like the ability to enter the host, evade the host defense system and grow by escaping host immune response (Cunliffe, 2008). These factors can be secretory, membrane-associated, or cytosolic in nature (Liu *et al.,* 2021). The bacterial adhesion and host cell evasion is enhanced by membrane associated virulence factors. The *Enterobacter* species have flagella and possess motility. This flagellum is involved in several other functions for example, biofilm formation, protein export, and adhesion (Haiko & Westerlund-Wikström, 2013). *Enterobacter* spp. also exhibits different types of endotoxins (Sanders & Sanders, 1997). Barnes *et al,* (2001) observed that in vitro *Enterobacter* spp. strains secreted enterotoxins, alpha-haemolysins, and cytotoxins like Shiga-like toxins II (Krzymińska *et al.,* 2009). In Gram-negative bacteria, a major pathogenicity factor includes the type III secretion system (TTSS). A study reported, 27% of *Enterobacter* spp. had TTSS as virulence factor (Paauw *et al.,* 2009). Secretory components are principle character of bacterial defence mechanisms that help bacteria to escape from immunological responses of the host (Sharma *et al.,* 2016).

Disease is not usually an outcome of a single virulence factor but depends mainly on the host susceptibility and microbe characteristics as virulence is a multifactorial phenomenon (Tarchouna *et al.,* 2013). Enterobacteriaceae group harbour multiple different virulence factors linked with virulence genes associated with serious infections (Naboka *et al.,* 2021). Such factors include adhesions (fimbriae (fimA and fimH), P-fimbriae (Pap operon), and S-fimbriae(s*f*a)), toxins (hemolysin (HlyA) and cytotoxic necrotizing factor (cn*f*-1)), iron acquisition system (Yersiniabactin (fyuA), Aerobactin (aer/iuc), and Enterobactin (entB)), hydrolytic enzymes (urease) and biofilm development (Wilson *et al.,* 2019).

2.10.1.1 Adhesins

The microorganism capable of attachment to host surface possess adhesins during subsequent colonization by biofilm formers. Bacteria have ability to attach to different biotic and abiotic surfaces such as medical surfaces like catheter, to develop complex biofilm structures. A study reported that *Enterobacter* spp. have adhesins which are Pilli and fimbriae (FimH) which aids in attachment to the surfaces (Govindarajan & Kandaswamy, 2022).

2.10.1.2 Fimbriae

Fimbriae (synonym: pili) are adhesins which are straight, stiff organelles distinct from flagella, are found in most Enterobacteriaceae family. Pilli are short appendage found in different bacterial species and can be a marker for determination of virulent strains. Because they are involved in development of infection by attachment to host cells (Shadan *et al.,* 2023). Fimbriae are involved in both enterotoxigenic and uropathogenic infections. Enterotoxigenic strains possess fimbriae that induce adhesion to the epithelial cells of the GI tract (Roberts *et al.,* 2004). All Gram-negative bacterial species possess one or more forms of fimbriae (Klemm *et al.,* 2010). For complete structure and assembly, they are regulated by cluster of genes. Major components that mediate attachment to host cell surface include FimA and FimH (Berne *et al.,* 2015). Clumping of RBCs also involves fimbriae adhesins that occur both in presence and absence of mannose (Kammili *et al.,* 2013). Type 1 fimbriae play role in biofilm formation and type 3 fimbriae promote biofilm formation, so are involved in infections (Berne *et al.,* 2015; Romero *et al.,* 2016). P-fimbriae (Pyelonephritis associated pili-operon) play a role in colonization by binding glycolipids on human erythrocytes of P blood group with α-D-galacopyranosy-(1-4) β-D-galactopyranside receptor epitopes (Klemm *et al.,* 2010). P-fimbriae are highly known for the long-term colonizer of GI tract (Johnson & Russo, 2002). However, S-fimbriae identifies alpha-sialyl-w,3-galactose receptors on glycoproteins of host cell and its

involvement in virulence and its molecular characterization remains unclear so far (Klemm *et al.,* 2010).

2.10.1.3 Biofilm Formation

Biofilm is the prevalent form of life for bacteria in a nutrient-reliable ecosystem. There are many factors for biofilm formation and adhesion to surface triggers it (Kramer & Assadian, 2014). This adhesion makes bacteria tolerant to various antimicrobials (Matthes *et al.,* 2013). Another reason for such tolerance includes the production of extracellular substances after attachment to abiotic surfaces like polysaccharides, proteins, and DNA. Biofilms have capacity to retain nutrients and water for protection of bacteria from external environmental (Flemming & Wingender, 2010). This is the reason biofilms are considerable persistence factor of microorganisms on inanimate surfaces in natural habitat and in industrial or medical areas (Donlan, 2002). This persistence duration of microbes on abiotic surfaces can be extended, depending upon the environmental conditions like humidity. Studies have reported biofilms on hospital area surfaces like supply buckets, opaque plastic doors, sink rubbers, and venetian blind cords (Vickery *et al.,* 2012). These types of microorganism have greater chances of exchanging genetic material such as virulence factors among their own species or other species which may be present in specific biofilm area (Tribble *et al.,* 2012).

2.10.1.4 Siderophores (Iron Acquisition System)

Iron plays a key role in engagement of several cellular proteins in different functions such as cellular respiration, production and maintenance of DNA, biodegradation, and biosynthetic pathways, and as a cofactor in transcriptional control (Evstatie & Gasche, 2012). The primary source of iron is diet for indigenous organism. This non-heme iron is incorporated into gastrointestinal duodenum ranges from 5 to 15%, while the rest unabsorbed iron leaves the body (Stein *et al.,* 2010). There are multiple mechanisms of bacterial iron acquisition, which includes siderophore mediated transport (Miethke & Marahiel, 2007; Sheldon & Heinrichs, 2015). This major feature of iron accumulation is linked with pathogenicity of bacteria present in the host environment and engaged in extraintestinal diseases. During infection, the iron excess for the microorganism is limited by mammals via alteration in their metabolic processes. The only microbe thrives and reproduce in the host which have access host iron. Therefore, establishment of iron acquisition mechanism in bacteria is necessary in infected tissues. The siderophore production is one of the bacterial strategies to acquire iron. Siderophore extract iron from ferrated transferrin and lactoferrin (Mokracka *et al.,* 2004). Depending on the functional groups, there are three structural families of siderophores i.e., carboxylate,

hydroxamate and catecholate (Miethke & Marahiel, 2007; Holden & Bachman, 2015). Yersiniabactin (fyuA), Enterobactin and Aerobactin (aer/iuc) are majorly produced siderophores by different Gram-negative bacteria including specie *Klebsiella, Enterobacter* and *E. coli* (Daoud *et al.,* 2022; Klebba *et al.,* 2021).

2.11 Virulence Assessment of genus *Enterobacter*

Within this genus *Enterobacter,* visible differences in pathogenicity can be observed among different species for instance, *E. aerogenes* and *E. cloacae* as they differ in the presence of gene encoding virulence factor. *E. aerogenes* also possess genes identical to *Klebsiella pneumonia* (Azevedo *et al.,* 2018). Also, genes responsible for bacterial adhesion and formation of biofilms like the fimH which encode adhesins of type 1 and mrkD genes which encodes type 3 fimbriae, are detected. It indicates that there is high number of bacterial virulence factors present in genus *Enterobacter* (El Fertas-Aissani *et al.,* 2013). Also, virulence factor like iron acquisition and transport, genes like kfu, entB, and ybtS, which regulate siderophores production have been identified in *E. aerogenes* too (Compain *et al.,* 2014). The specie *E. hormaechei* is reported as most virulent than other species because of presence of a high-pathogenicity island (HPI) which can be detected on its chromosome. This HPIs also contains siderophore-encoding genes, such as irp2 gene which has been determined in *Enterobacter spp.* (Souza Lopes *et al.,* 2016). The bacteria possessing virulence may have the ability to assimilate iron through chelators, necessary for metabolism and establishment of infection. ECC strains or subspecies possess curli-encoding genes which play role in host cell adhesion and invasion.

Chapter 3: Materials and Method

3.1 Study Design

This cross-sectional study was conducted at Molecular Microbiology Laboratory, Department of Microbiology, (Quaid-i-Azam University, Islamabad) from August 2022 to July 2023. A total 52 Food samples and 73 Abiotic samples were collected from different mass gatherings of Pakistan. All the standard microbial techniques and methods were used to identify and characterize the isolates.

3.2 Study Site

Total of 52 Food samples were collected from different mass gatherings of Islamabad, Lahore, Sargodha and Attock, Pakistan. Majority 73 of the abiotic samples were collected from regular mass gatherings of Islamabad, Pakistan.

3.3 Survey

Data was collected through survey based questionnaire comprising of various section on; type of event (indoor or outdoor), duration of event (1 day, 2 days or more), demographic parameters (gender, age group of attendees, nationality, marital status and socioeconomic level), location, security level, behavioural attributes (maintenance of cleanliness), hygiene facilities (washroom facility, handwashing facility, food handlers wearing caps and gloves, waste disposal facility available. In addition, the way food was prepared, stored, and served during the events was recorded.

3.3.1 Inclusion Criteria

Mass gathering with attendees ranging from 300 to 1000 were included in the study. Food sample and abiotic samples were taken from the mass gathering.

3.3.2 Exclusion Criteria

No gathering less than 299 individuals was included in the study. Along with that sampling from hotel and restaurants were excluded.

3.4 Sampling

For sample collection, there were two main categories of sampling using a systematic sampling approach as follows:

1. Regular Mass Gathering Events included soup kitchens (once per month from 3 different locations for six months)
2. Special Mass Gathering Events (single point sampling per event)

3.4.1. Regular MG events

Regular MG events include those mass gatherings that happened periodically throughout the year. Monthly sampling from three different soup kitchens in Islamabad was done from mid-August 2022 to December 2022. The food samples were collected at food distribution time. Different cooked food samples were collected which included meat and poultry, rice, vegetables, and pulses. The three Soup Kitchens are in various sectors of Islamabad which are given codes as follows:

• RP, Islamabad at G-8

• RG, Islamabad at G-9

• RI, Islamabad at I-11

3.4.2. Special MG Events

Samples were collected from the planned occasional events occurred in Islamabad, Lahore, and Rawalpindi. These included cultural festivals, religious event, music concerts, food festivals, university fairs, and Iftar party. Ready to eat food samples were collected from different stalls present at the event. Food samples included were barbequed chicken/meat, golgappas, pan, Channa chat, chowmain and noodles. These events are enlisted in Table 3.1.

Table 3.1 Special MG events covered from August 2022 to December 2022

Sr. no	Date	Event Name	Location
1	8-Aug-22	Ashura	Sargodha
2	8-Aug-22	Ashura	Attock
3	20-Aug-22	Evening With Crafts	Islamabad
4	9-Oct-22	Rabi-ul-Awal	Sargodha
5	9-Oct-22	Rabi-ul-Awal	Islamabad
6	16-Oct-22	Saqafat Cultural Display	Islamabad
7	29-Oct-22	Halloween Family Festival	Islamabad
8	11-Nov-22	Food Fest Ayub Park	Rawalpindi
9	7-Dec-22	Lok Mela	Sargodha
10	25-Dec-22	Women's Gala	Islamabad

3.5 Sampling Technique

3.5.1 Food sampling

Food sampling was done according to the procedure provided by the Food and Drug Administration (FDA). Almost 25-30 grams of each food sample were collected in sterile containers aseptically. During sampling, hands were sterilized properly. The size of container must be suitable for adequate collection of food material and plastic must be leak-proof. All samples were marked and labelled properly before transportation. All the samples were transported under refrigerated condition using ice box to the Molecular Microbiology Laboratory, Department of Microbiology (Quaid-i-Azam University) for further processing.

3.5.2 Abiotic Surface Sampling

Surface sampling was performed via sterile cotton swabs. Areas to be swabbed included benches, table, and kitchen utensils (jug, plate, glass). Approximately 10 x 10 cm of the area was swabbed in square for 1 minute. After swabbing the surface, these cotton swabs were carefully placed in Eppendorf having 1 % normal saline solution. Each sample was labelled properly. All the samples were transported under refrigerated condition using ice box to the Laboratory for further processing.

3.6 Sample Processing

Aseptic techniques were followed during sample handling. The sample containers were cleaned with 70 % ethanol before processing to avoid the cross contamination of sample.

3.6.1 Sample Preparation of Food Samples

Twenty-five grams of each food sample was weighed and transferred into sterile polythene zip lock bags. The sample in each sterile bag was then mixed thoroughly with 225 mL of buffered peptone water. This mixture was homogenized to obtain a uniform mixture which served as stock.

3.7 Sample Culturing and Quantitative analysis

3.7.1 Enrichment of Food Samples

For enrichment, 1 gram of homogenized food sample was added in to 9mL of buffered peptone water. The sample was then incubated at 37°C for 24 hours. After incubation, a loopful of inoculum was streaked in a quadrant manner on a nutrient agar plate and incubated at 37°C for

24 hours. After incubation, plates were observed for the presence of different types of colonies. Colonies were selected based on their morphological characteristics like size, colour, margin, shape, and elevation. The selected colonies were re-streaked on a fresh nutrient agar plate for purification. Single pure isolates obtained were subjected to further testing for identification.

3.7.2 Plating of Abiotic Surface sample

The abiotic samples were vortexed for 20 seconds. Afterwards, 0.1mL from sample was spread onto nutrient agar plates by glass spreader following spread plate method. The plates were then incubated for 18-24 hours at 37°C in incubator.

3.8 Preliminary Identification of Isolates

3.8.1 Isolation and Purification of Bacterial Culture

Different colonies were observed on nutrient agar plates with distinct morphology. Each of these distinct colonies were picked by sterile inoculating loop and streaked onto fresh nutrient agar plates separately, following quadrant streaking method. The plates were then incubated for 18-24 hours at 37°C in incubator. After incubation, bacterial colonies were purified by re streaking onto agar plates and were incubated. The pure isolates obtained were subjected to further testing for identification.

3.8.2 Gram staining

The Gram staining technique differentiates the bacteria into two distinct groups, Gram-negative and Gram-positive. The principle lies on the structural differences of the bacterial cellular membrane and cell wall. Gram-positive organisms contain rich peptidoglycan which retains the crystal violet-iodine complex (CV-I) and gives purple colour. Gram negative microorganisms have thin peptidoglycan; hence CV-I complex is not retained secondary stain safranin and give pink colour.

3.8.2.1 Smear Formation

On a sterile slide drop of normal saline or distilled water was added in centre of marked circle using the dropper., Using sterile inoculating loop or toothpick, a single fresh bacterial colony (18-24 hours) was picked from agar plate and was mixed with drop of water to form bacterial smear. Leave the slides to air dry and were then heat fixed using spirit lamp.

3.8.2.2 Staining Procedure

On a heat fix slide, crystal violet was added for 60 seconds. The slide was then washed, and Gram's iodine was dropped for 60 seconds. Again, slide was washed, and decolourizer was added for 5 to 6 seconds. Rinse the slide with tap water. Afterwards counterstain safranin was dropped for 60 seconds. The slides were then washed and left to air dry.

3.8.3 Microscopic Examination

After staining, bright-field microscope was used for observation. The slides were then observed under 100 X objective lens with immersion oil. An immersion oil increases visibility and provide more details on the structure, and staining features.

3.9 Biochemical Identification

3.9.1 Purification of Gram-negative bacteria

After Gram staining, all Gram-negative bacteria were identified and were streaked onto MacConkey agar plates as it is selective media for Gram negative bacteria. The media is based on the principle of lactose fermentation. 51.5g/L MacConkey agar media (Sigma-Aldrich) was prepared with distilled water and autoclaved at 121°C and 15 psi for 20 min. Gram positive bacteria do not show growth due to the presence of crystal violet and bile salts in the media. The streaked plates were incubated for 18-24 hours at 37°C. After incubation period, lactose fermenters having pink colour colonies were differentiated from non-lactose fermenter which were colourless colonies.

3.9.2 Biochemical Testing

Microorganisms were further identified on the basis of series of biochemical tests for *Enterobacteriaceae,* which was further compared with Bergey's Manual for the identification of *Enterobacter spp.*

3.9.2.1 Catalase Test

This test was performed to detect presence of catalase enzyme. Catalase enzyme is responsible for degradation of hydrogen peroxide, and reaction is as follow:

$$2H_2O_2 \xrightarrow{\textbf{Catalase}} 2H_2O + O_2 \uparrow$$

Catalase production was determined by slide method. Drop of 3 % H_2O_2 was added on the slide, with the help of autoclaved toothpick single colony from culture was mixed with the 3% hydrogen peroxide. The formation of bubbles indicates positive catalase test, whereas no formation of bubbles indicates catalase negative test.

3.9.2.2 Oxidase Test

The principle of this test is that some bacteria oxidise dimethyl-p-phenylenediamine and α-naphthol to generate indophenol blue in the presence of enzyme cytochrome oxidase. Kovac's reagent (tetra-methyl-p-phenylenediamine dihydrochloride, 1%) was used, which serve as substrate that donate electron and oxidize to black compound in presence of oxidase enzyme. Drop of reagent was added on filter paper. Next with help of sterile toothpick a colony was picked and mixed with oxidase reagent. The colour change from pink to dark purple colour indicated presence of oxidase enzyme and gives positive result. No change in colour was indicated as negative result.

3.9.2.3 Sulphide Indole Motility Test

a. Indole Production Test

Indole serve as a biochemical marker and is generated in enzymatic process, where an amnio acid tryptophan is oxidized with the help of enzyme tryptophanase**.** This experiment needs SIM agar which poses tryptophan as substrate. SIM media was prepared according to manufacturer's instruction and autoclaved at 121℃. After autoclaving, 5mL of media was poured into the test tubes. Using aseptic method, the culture was picked from plate using sterile inoculating loop and stabbed in the SIM agar tubes to the depth of 1-2cm to the bottom of the tube. After inoculation the tubes were incubated at 37℃ for 24 to 48 hours. Next, 10 drops of Kovac's reagent was added in tube cultures and observation was made after few minutes. The culture which gave red layer were indole positive.

a. **Hydrogen Sulphide Production Test**

For this test the SIM media composed of peptone and sodium thiosulfate is used. The media contains an indicator, ferrous ammonium sulphate. The media was prepared in distilled water and autoclaved at 121℃ for 20 minutes. In aseptic conditions, media was poured in tubes and stab inoculation was performed by picking pure colony from the plate using sterile inoculating loop. The tubes were incubated at 37℃ for 24 hours. This forms an insoluble black ferrous sulphide precipitate along the line of the stab inoculation giving a positive test result. If there is no black precipitate this indicate as negative test result.

b. Motility Test

To detect motile microorganisms, semi solid medium is used. If the microorganism is motile, the bacterial growth will be radiated from stab line. Motile bacteria also make media turbid. If bacterial growth is confined to stab line this will indicate bacteria is non-motile. SIM media was prepared according to manufacturer's instruction and autoclaved at 121℃. After

autoclaving, 5mL of media was poured into the test tubes. Using aseptic method, the culture is picked from plate using sterile inoculating loop and stab in the SIM agar tubes to the depth of 1-2cm to the bottom of the tube. After inoculation the tubes were incubated at 37℃ for 24 to 48 hours.

3.9.2.4 Citrate Utilization Test

This test was performed to determine citrate permease enzyme which facilitates citrate transport inside cell. This enzymatic reaction produces alkaline product, sodium carbonate, due to production of carbon dioxide. The bromothymol blue indicator detects sodium carbonate in media and gives a colour change from green to deep Prussian blue. Simmons citrate agar was prepared according to manufacturer's instruction and autoclaved at 121℃ for 20 minutes. The autoclaved media was poured in test tube under aseptic conditions and agar slants were made. The sterile inoculating loop was used to pick the culture from plate and was streaked onto agar slants. The tubes were incubated for 24 hours at 37℃ and results were observed.

3.9.2.5 Methyl Red Test

This test is used to detect acid production by glucose fermentation. Glucose is major hexose monosaccharide, which is utilized for energy production by all enteric microorganisms, but the end-product generated by this enzymatic reaction depends on the pathway being utilized by bacteria. The MR-VP broth is used as media for this reaction. The MR-VP broth was prepared according to manufacturer's instruction and autoclaved at 121℃ at 15 psi for 20 minutes. The autoclaved media was poured in tubes under aseptic conditions and culture was inoculated using the sterile inoculating loop. After inoculation, labelled tubes were incubated for 24 hours at 37°C. Afterwards, one-third culture was separated into another tube for Voges-Proskauer test. Methyl red indicator detects the concentrations of organic acids generated as end products. On acidic pH media will turn red and indicates positive test. However, no colour change shows negative test. After 24 hours, five to seven drops of the methyl red indicator were added into each incubated tube and results were observed.

3.9.2.6 Voges-Proskauer Test

The Voges-Proskauer test differs from methyl red test in detecting the presence of nonacidic end products. In this test, Barritt's reagent is used for detection of nonacidic end products. Barritt's reagent A is a mixture of alcoholic α-naphthol and Barritt's reagent B is 40% potassium hydroxide solution. This biochemical reaction results in a pink complex, imparting rose colour to the medium, indicating positive test.

The one-third culture separated during methyl red test was used for Voges-Proskauer test. Firstly, Barritt's reagent A (10 drops) were added, and tube was shaken gently, then 12 drops

of Barritt's reagent B were added and tube was shaken. The results were noted after 3 to 4 minutes.

3.9.2.7 Triple sugar-iron Test

The triple sugar–iron (TSI) agar test distinguishes different genera of the Enterobacteriaceae on the basis of carbohydrate fermentation and hydrogen sulphide production. The media contains 1 % lactose, 1% sucrose and 0.1% glucose (dextrose) with acid-base indicator, phenol red. This medium also contains sodium thiosulfate, which act as a substrate to generate hydrogen sulphide (H_2S) and ferrous sulphate. TSI agar media was prepared according to manufacturer's instruction and autoclaved at 121ºC at 15 psi for 20 minutes. The media was poured in test tubes and slants/butt were made. Stab and streak procedure was followed for the inoculation of bacterial culture. Results observed were of three different types after incubation period of 18-24 hours at 37ºC.

3.9.2.8 Urease Test

The principle of this test is to detect the production of urease enzyme by bacteria. This enzyme act on amide compounds, breaking down the nitrogen and carbon bond and forms end-product, ammonia. The urea broth medium detects the presence of urease by pH indicator phenol red. In the presence of alkaline end product, media colour changes from yellow to deep pink colour. This colour change is a positive result, whereas no colour change indicates a negative result. The media was prepared as per manufacturer instructions, after autoclaving at 121°C media was poured in test tubes. Using aseptic technique, the culture was inoculated by sterile inoculating loop and was incubated at 37°C for 24 hours. After 24 hours, the results were observed.

3.10 Purification of *Enterobacter* specie on differential media

After the biochemical testing, the *Enterobacter spp.* was confirmed on differential media. Eosin methylene blue (Levine) agar contains dyes eosin and methylene blue in ratio 6:1 as colour indicators along with other components. This medium allows growth of gram-negative bacteria and partial inhibitory growth of gram-positive bacteria. The presence of indicators along with lactose in medium differentiate between enteric lactose fermenters and non-lactose fermenters. *Enterobacter spp.* produce pink-purple colonies on this medium. The media was prepared in distilled water according to manufacturer's instruction and autoclaved at 121ºC for 20 minutes. Under the aseptic conditions, media was poured in plates and were labelled properly. The plates were streaked with sterile inoculating loop. After streaking, plates were incubated at 37ºC for 18-24 hours in incubator.

3.11 Phenotypic Determination of Virulence Factors

A total 23 newly identified *Enterobacter* spp. from food samples and abiotic samples along with 27 previously isolated and identified *Enterobacter* spp. from MGs were processed for determination of their pathogenic and virulence potential. These 27 isolates were revived from glycerol stocks and were confirmed as *Enterobacter* spp. before processing.

3.11.1 Hypermucoviscosity Assay

String test is performed to check the hypermucoviscosity of *Enterobacter* spp. Fresh culture of pure isolates of was grown on EMB agar plates and were touched by sterile inoculating loop which was slightly raised to observe their capacity to form string. The positive result was depicted only if the string was greater than 5mm in length.

3.11.2 Haemolysin Assay

Haemolysin production assay is based on lysis of red blood cells (RBCs). There are three types of haemolysins depending on lysis capability which are alpha (α), beta (β) and gamma (ϒ). Beta-haemolysin results in complete lysis of RBCs, making clear zones near bacterial colonies. α-haemolysin results in partial lysis of RBCs and ϒ-haemolysin causes no lysis of RBCs. For this assay, blood agar base was used and 5% defibrinated sheep blood was mixed with it. Blood agar base (40g/1000mL, Oxoid) was prepared and autoclaved at 121℃ for 20 minutes and 15 psi pressure. After the media cooled down to 45°C, aseptically 5% defibrinated sheep blood was mixed in media. After pouring media into plates, isolates were streaked on sheep blood agar plates using sterile inoculating loop. After streaking, plates were incubated at 37℃ for 24 hours and results were observed after 24 hours.

3.11.3 Biofilm Assay

Two different types of assays were performed on *Enterobacter* isolates for biofilm detection. One was qualitative assay, referred as Congo red assay and other is quantitative assay referred as Microtiter plate assay.

3.11.3.1 Congo Red Assay

Congo red assay determine the biofilm formation ability of bacterial isolates. For this assay, the Congo red agar (CRA) was prepared through the components mentioned in Table 3.2. The media components were autoclaved separately whereas Congo red dye was autoclaved separately for 15minutes at 121℃ and 15 psi. After that, dye was mixed with the media. When media was cooled down it was poured into plates and were labelled properly. Isolates were streaked down by using sterile inoculating loop on CRA plates. The results were observed after 24 hours of incubation.

Table 3.2: Essential Components of Congo Red Agar Media

Typical Formula	**g/L**
Sucrose	36.0
Brain Heat Infusion (BHI) Broth	37.0
Congo Red Dye	0.8
Agar-Agar	10.5
Distal Water	1000
pH 7.1 ± 0.2 at 25℃	

3.11.3.2 Microtiter Plate Assay

Microtiter plate assay was performed on *Enterobacter* isolates to determine their biofilm formation ability. The protocol followed was divided into 3 steps as follow:

a. Development of biofilm

The inoculum for assay was prepared in Brain Heart Infusion (BHI) broth and incubated overnight at 37℃. The turbidity of the inoculum was adjusted with 0.5% McFarland's standard. Afterwards, 200µL of grown culture was taken into 96 well microtiter plate (MTP) and incubated for 24 hours at 37℃. Wells containing only sterile BHI broth were taken as negative control whereas wells with biofilm forming strain were taken as positive control.

b. Washing and staining

Following incubation, the microtiter plate was tapped gently to remove the media from wells without disruption of biofilm. MTP was washed with 200µL of phosphate buffer saline (PBS) to remove the unattached bacterial cell components. After this, 30 minute of incubation was followed with addition of 200 µL of 95% ethanol. The biofilms were stained with 1% of crystal violet and left for 15 minutes. MTP was rinsed twice with autoclaved distal water or PBS to remove excessive dye. The plate was incubated at room temperature for 30 minutes to dry completely. A 200µL of 33% acetic acid was added and after 15 minutes of incubation OD was taken using ELISA reader.

c. Quantification of biofilm

Optical density was measured at 600 to 650 nm for quantification of biofilm and compared with cut off value. The cut off value (ODC) was determined using following formula:

ODC= average OD of the negative control + 3(standard deviation of negative control)

The results were noted using the criteria mentioned in Table 3.3.

Table 3.3: Criteria for Detection of Biofilm Formers by MTP Method

Weak Biofilm Producer	OD= 2(ODC)
Moderate Biofilm Producer	2(ODC)≤OD=4(ODC)
Strong Biofilm Producer	OD≥4(ODC)

3.11.4 Haemagglutination Assay

This assay is characteristic feature of *Enterobacteriaceae*. The bacteria which possess type 1 fimbriae agglutinate the human blood red blood cells (RBCs). This test consists of following steps.

a. Collection and preparation of RBCs

The blood samples were taken from healthy individuals having different blood groups (Blood group A, B and AB) in EDTA tubes. RBCs were collected through centrifugation at 5,000 rpm for 5 minutes. The supernatant was discarded, and freshly prepared PBS with pH 7.2 was used to wash RBCs. Working solution of RBCs (3%v/v) was prepared to perform haemagglutination test.

b. Preparation of inoculum

Freshly grown bacterial culture was mixed in 5mL of BHI broth and incubated overnight.

c. Procedure

Centrifuge the inoculum at 5,000 rpm for 5 minutes. The supernatant was discarded, and bacterial cells were suspended in PBS to obtain the concentration of $5x10^{10}$ cells/ mL. A 50µL of bacterial suspension was added into each well of microtiter plate with 50µL of 3% v/v working solution of RBCs with different blood group. The MTP was incubated for 24 hours at 4°C. Results were observed by observing the clumping of RBCs.

Chapter 4: Result

4.1 Sampling

This cross-sectional study was conducted at Molecular Medicine Laboratory, Department of Microbiology (Quaid-i-Azam University) from August 2022 to July 2023. Total 52 Food samples and 73 Abiotic samples were collected from different mass gatherings of Islamabad, Lahore, Sargodha and Attock. There were 10 special MGs event which were food festivals (3), seasonal gala (3), and religious events (4), and 12 regular MGs events. From total 52 food samples, 30 food samples were collected from special MG events and 22 food samples were collected from regular MGs. Out of 73 abiotic samples, 8 sample were collected from special MG events and 65 samples were collected from regular MG events.

4.2 Sampling Site

A survey was conducted for each mass gathering site to collect data (Figure 4.1). For special MG events, sites were located in the city of Islamabad, Lahore, Sargodha, and Attock. It was observed that a large number of attendees were present in various MG events as shown in Figure 4.2. However, for regular MG events sites were all in the city of Islamabad.

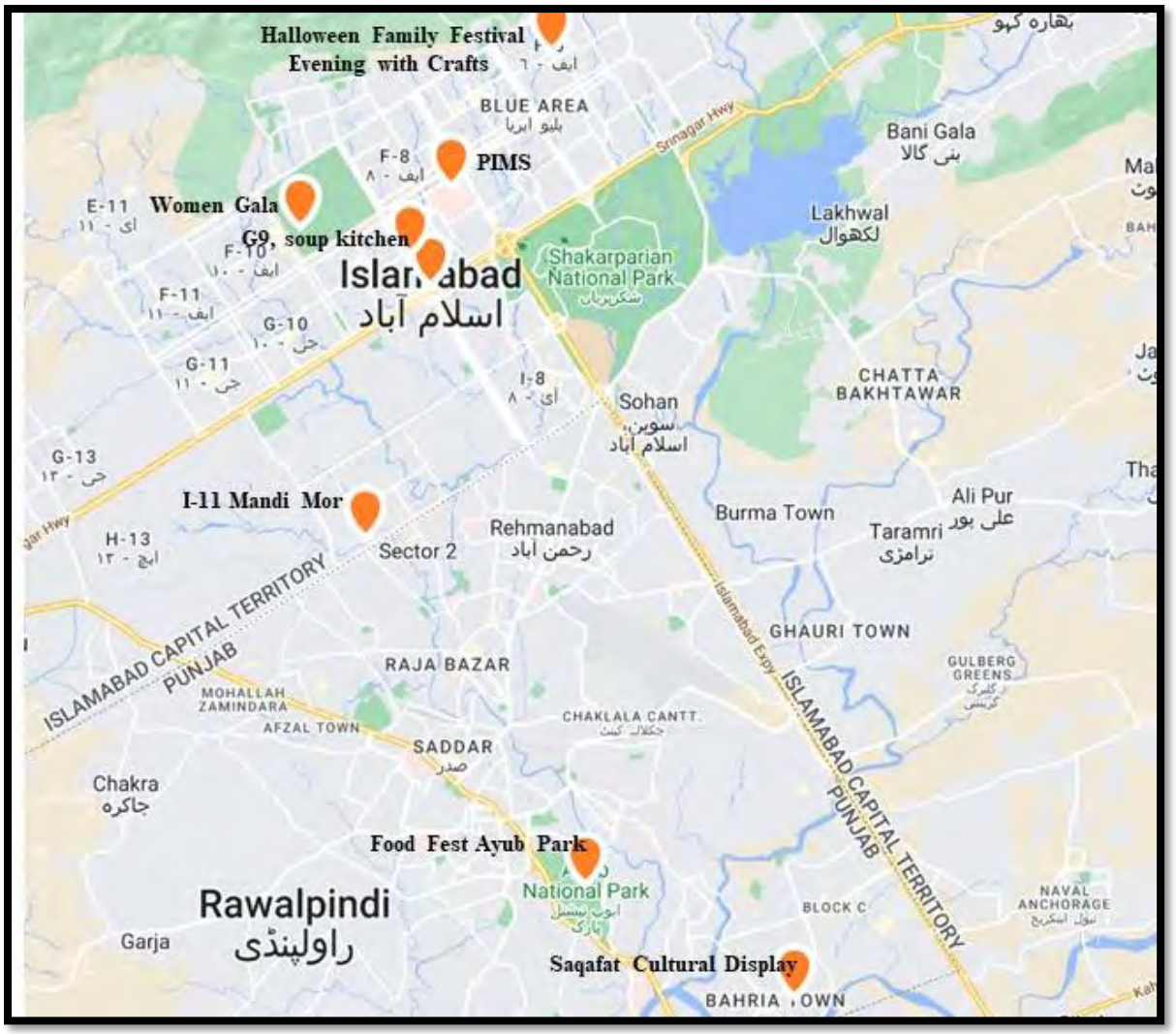

Figure 4.1 Map showing specific areas of different MG sampling sites from August 2022 to December 2022

4.3 Survey

Data was collected about MG events through observation-based questionnaire. The questionnaire was filled out for both special and regular MGs. Foreigners were observed in special MG events especially in food festivals and in seasonal gala. Risks factors regarding food borne illness were also recorded. In different special MG events, the raw and cooked food was not placed separately on a single stall, as shown in Figure 4.3. Also in special MG events, the food items were not covered as illustrated in Figure 4.4-4.5, no maintenance of cleanliness and no waste disposal facility was also available. Another important factor that was observed in MGs was that food handlers were not wearing hair nets and gloves as illustrated in Figure 4.6.

Figure 4.2 A view of mass gathering showing a large number of attendees present in Food Fest (special MG event) in Islamabad, on November 11,2022 at 7:30 pm.

Figure 4.3 Half cooked and uncooked chicken tikka placed on the same barbeque stand at a stall in a food festival of a special MG event.

Figure 4.4 Uncover food items placed in unhygienic conditions in Halloween Food Festival (special MG event)

Figure 4.5 Uncovered pan and other food items available in Food Festival Ayub National (special MG event)

Figure 4.6 Food handlers were handling food with bare hands in Lok Mela, Sargodha (special MG event).

4.4 Isolation of Bacterial species

All food samples, after homogenization were enriched in buffered peptone water, streaked on nutrient agar plates, and incubated at 37°C for 24 hours. Simultaneously, all abiotic samples were spread on nutrient agar plates by pour plate method and incubated at 37°C for 24 hours. Colony morphology of the isolates was noted after incubation. Based on different colony morphological characteristics (colour, opacity, shape, elevation, appearance of colony, and margin) isolates were given codes and treated as possible different bacterial strains. Each distinct colony isolated was further streaked onto a nutrient agar plate for purification (Figure 4.7) and was incubated for 24 hours at 37°C. From 52 food samples, a total 83 Gram positive cocci and negative rods as coccobacilli were obtained. However, from 73 abiotic samples 142 bacterial isolates were purified.

Figure 4.7 Purified bacterial colonies of isolate LF-181 after quadrant streaking on nutrient agar after incubation for 24 hours at 37℃.

4.5 Preliminary Identification of Isolates

4.5.1 Gram Staining

All the isolates obtained from food samples and abiotic samples were subjected to Gram staining to differentiate between Gram-positive and Gram-negative bacteria (Figure 4.8). Out of 83 isolates of food samples, 50 isolates were Gram-negative rods and 33 were Gram-positive cocci. Out of 142 isolates from abiotic samples, 67 isolates were Gram-negative rods, and 75 isolates were Gram-positive cocci. Only Gram-negative rods obtained from both food samples and abiotic samples were proceeded further for biochemical identification.

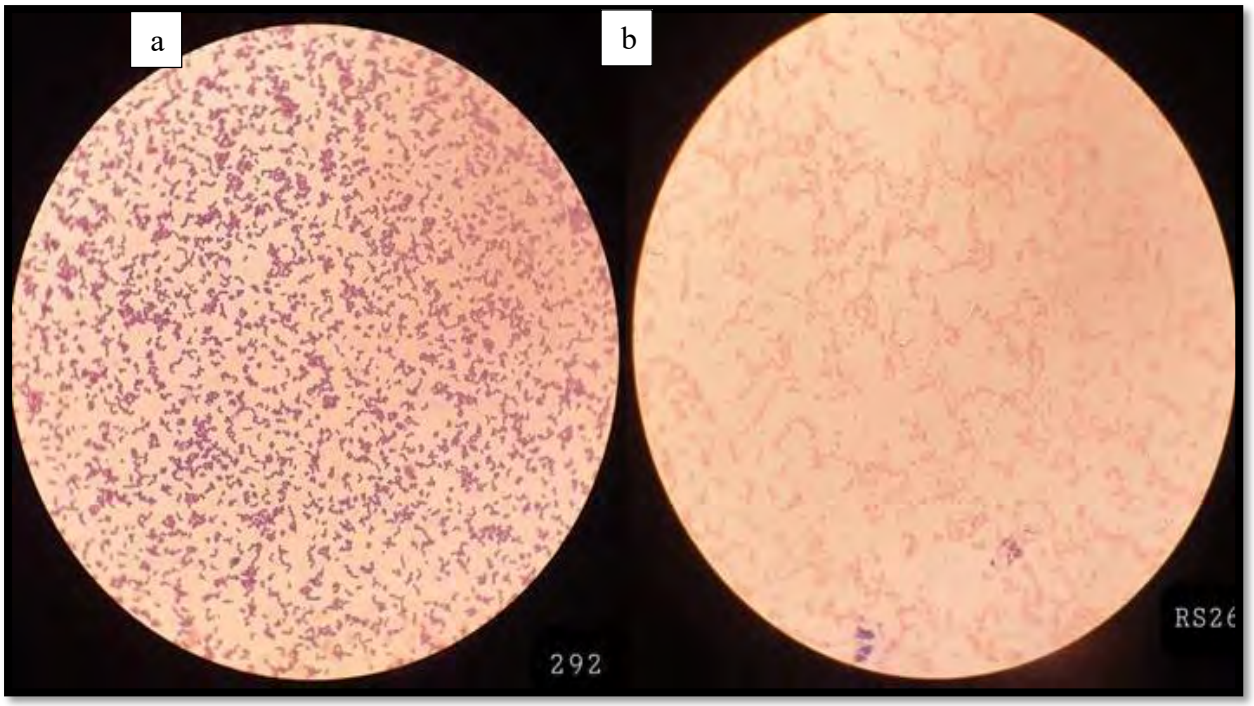

Figure 4.8 Morphology of the isolates under microscope (100X) after Gram staining (a) Gram-positive cocci RS 292 (b) Gram-negative rods RS 262

4.5.2 Isolation of Gram-Negative Rods

All the Gram-negative isolates were streaked on the MacConkey agar plates and incubated at 37°C for 24 hours to differentiate lactose fermenters from non-lactose fermenters. The *Enterobacter* spp. is a lactose fermenter, giving red to pink colonies on MacConkey agar plates. Out of 117 Gram negative rods, 48(41%) isolates were lactose fermenters while 69(59%) isolates were non-lactose fermenters.

From food samples, out of 50 Gram negative rods, 30(60%) were lactose fermenters while 20(40%) were non lactose fermenters. In case of abiotic samples, out of 67 Gram negative rods 18(27%) were lactose fermenters while 49(73%) were non lactose fermenters (Figure 4.9). Among lactose fermenters, isolates showing colony morphology pink, mucoid, raised were suspected as *Enterobacter* isolates, which were further subjected to biochemical characterization for identification.

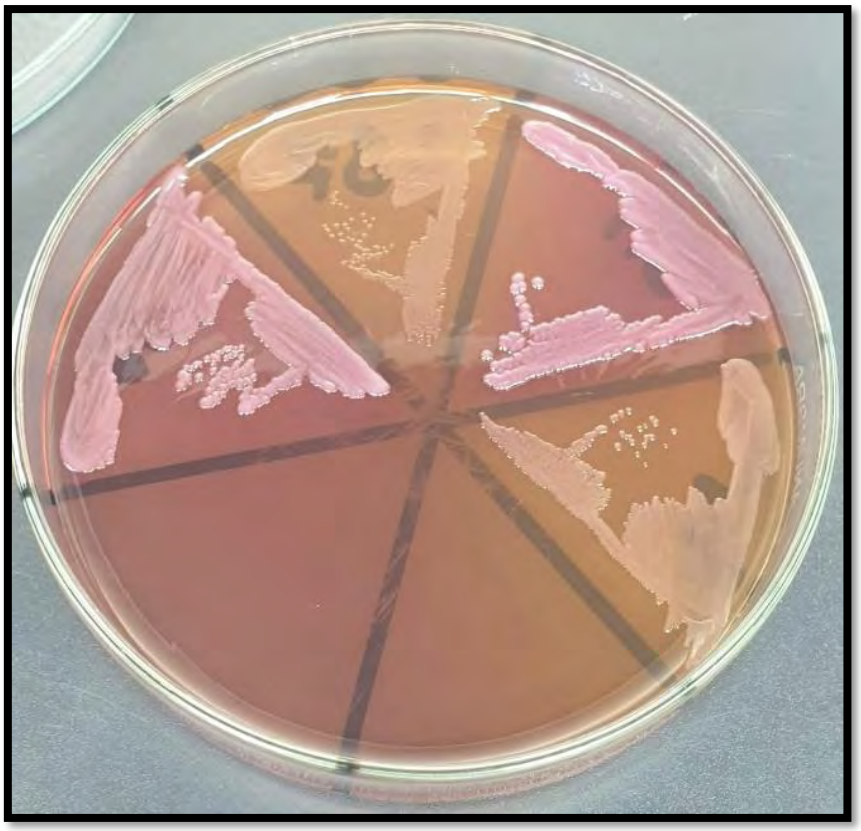

Figure 4.9 Growth of Lactose fermenter (pink colonies) of isolates LF 122, LF 123 and Non-lactose fermenters (yellow colonies) of isolates LF 160, LF 166 on MacConkey agar plate after 24 hours of incubation at 37℃

4.6 Biochemical Characterization of *Enterobacter spp.*

4.6.1 Catalase Test

This test was performed on all Gram-negative rods to identify the presence of catalase enzyme. All isolates showed positive result for catalase enzyme (Figure 4.10).

Figure 4.10 Catalase test for isolate LF 84 showing positive results by the formation of gas bubbles due to breakdown of H_2O_2.

4.6.2 Oxidase Test

The principle of this test lies in production of indophenol blue by certain bacteria from the oxidation of dimethyl-p-phenylenediamine and α-naphthol. Out of 117 Gram negative isolates, 111(95%) isolates were oxidase negative, and 6(5%) isolates were oxidase positive (Figure 4.11).

Figure 4.11 Oxidase test showing oxidase negative results (no violet colour) for isolates LF84 and LF93.

4.6.3. Sulphide Indole Motility Test

4.6.3.1 H_2S Test

H_2S test determined whether the bacteria could reduce the sulphur containing compounds to sulphides during the process of metabolism. As a result of reduction process, hydrogen sulphide gas was produced which is indicated by the blackening of the media (Figure 4.12). Out of 117 Gram negatives, 5 (4%) isolates produce H_2S while 112(96%) didn't produce H_2S gas.

4.6.3.2 Indole Production test

This test was performed to determine the tryptophanase activity of bacteria via Kovac's reagent. The positive results were indicated by appearance of a red-violet ring at the top surface of the test tube after the addition of Kovac's reagent. The *Enterobacter* spp. lacks the tryptophanase enzyme hence negative result will be given. Out of 117 Gram negative isolates, 15(13%) isolates were positive for the indole test and 102(87%) isolates showed negative results for indole test (Figure 4.12).

4.6.3.3 Motility test

The motility test differentiates motile bacteria from non-motile bacterial isolates. The motile isolates growth was radiated out of the stab line and non-motile isolates showed growth around the stab line. The *Enterobacter* spp. is motile and usually give radiated growth out of the stab line making the media turbid. Out of 117 Gram negative isolates, 25(21%) were negative for the motility test and 92(79%) were appeared as motile.

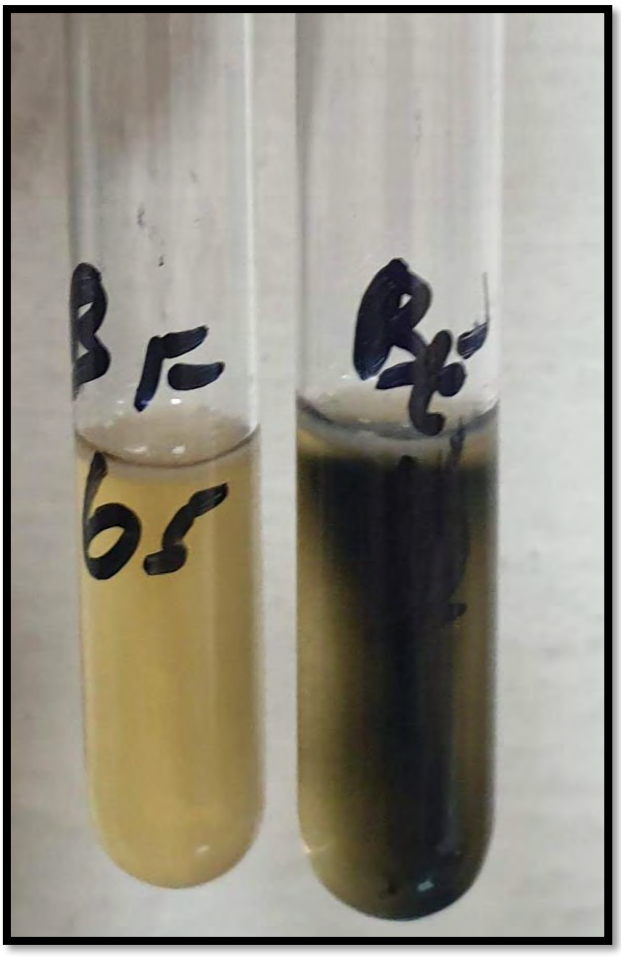

Figure 4.12 SIM test for Gram negative bacteria after incubation for 24 hours at 37℃, illustrate H_2S production of isolate BF66 showing a positive result by blackening of media and isolate BF 65 illustrating negative result for Indole by no ring formation.

4.6.4 Citrate test

Citrate test was performed to detect the citrate utilization ability of isolates. Colour change from green to blue illustrates positive results, whereas no change in media colour is indication of negative result (Figure 4.13). Out of 117 Gram negative isolates, 99(85%) isolates gave positive result for this test while 18(15%) gave negative result.

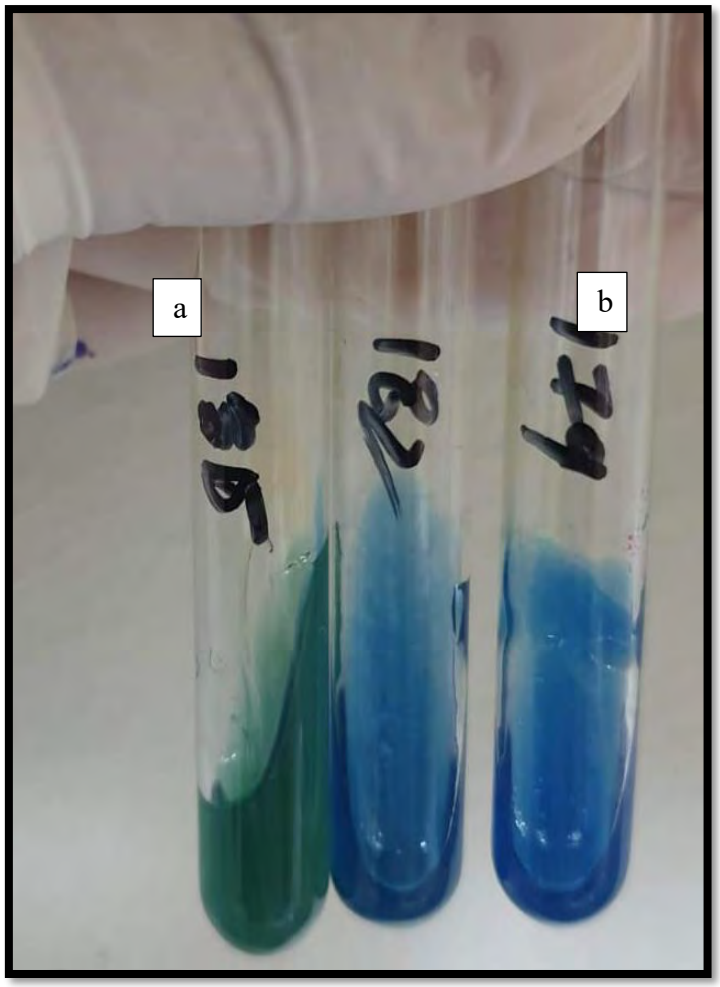

Figure 4.13 Citrate Utilization test on citrate agar media after incubation of 24 hours at 37°C, (a) isolate LF 185 illustrating positive result by colour change from green to blue (b) LF 182, LF 179 illustrating negative results by no colour change.

4.6.5 Methyl Red test

The methyl red test was performed to determine bacterial ability to utilize glucose by different pathways and differentiate between acid forming and acetoin forming bacteria. Upon the utilization of glucose, stable acidic products produced which turns the media colour from yellow to red. This red colour indicated positive result while yellow colour indicated negative result. The *Enterobacter* give variable results depending on type of specie. After performing this test on 117 Gram negative rods, 54(46%) isolates gave positive results, and 63(54%) isolates gave negative results (Figure 4.14).

4.6.6 Voges-Proskauer test

The Voges Proskauer test was performed to detect the non-acidic end products produced by bacteria after glucose utilization. After incubation formation of pink/red colour ring on top surface of the test tube indicates positive results. Out of 117 Gram negative isolates, 58(49%) isolates gave positive result for the VP test while 59(51%) were gave VP negative result (Figure 4.14).

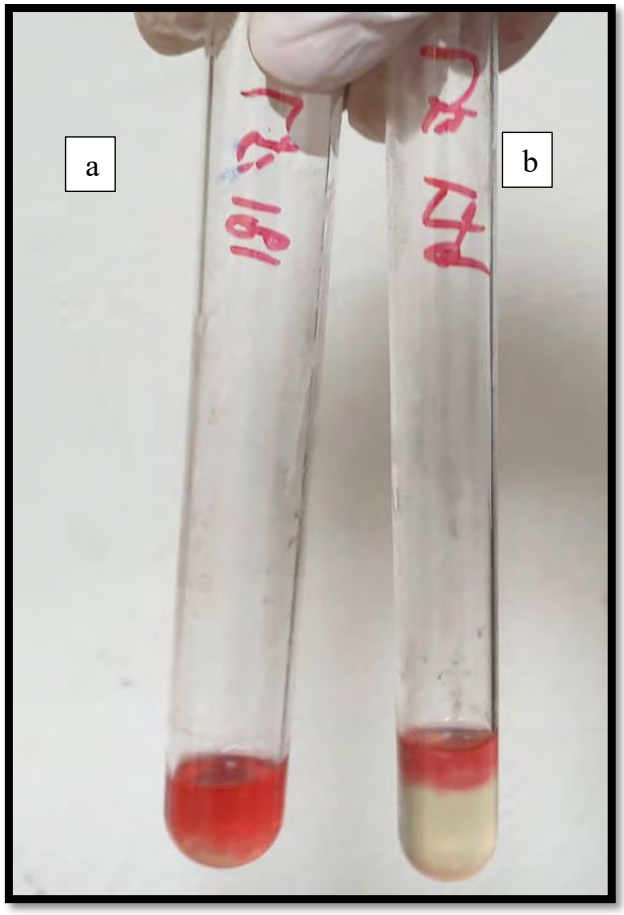

Figure 4.14 MR test and VP test on MR-VP broth media after incubation for 24 hours at 37°C (a) MR positive isolate LF 181 by the change in colour of media from yellow to red colour and (b) VP positive isolate LF 179 by showing cherry red colour ring.

4.6.7 Triple Sugar Iron test

This test differentiates bacterial isolates on their ability to ferment carbohydrates (glucose, sucrose, and lactose) and gas production. Among total Gram-negative isolates, acidic slant and acidic butt, alkaline slant and alkaline butt, alkaline slant and acidic butt, and alkaline slant and acidic butt with gas production was given by isolates. (Figure 4.16 and Figure 4.17).

Figure 4.15 Growth of isolate LF 191 on TSI agar media after incubation for 24 hours at 37°C, illustrating glucose fermentation with gas production resulting in alkaline slant and acidic butt with rising in media.

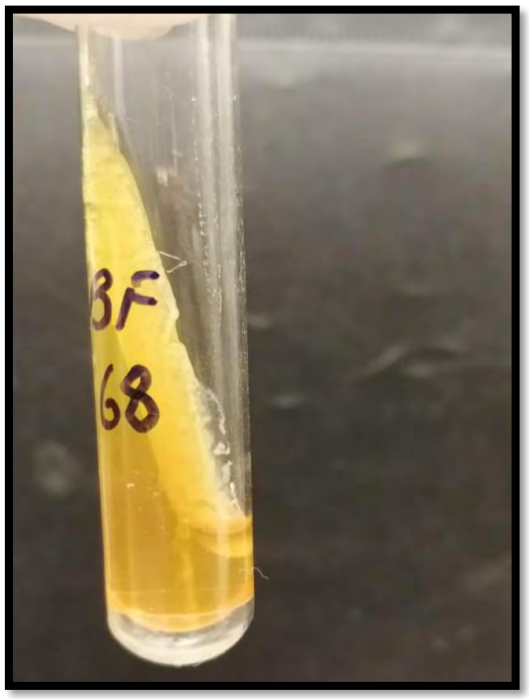

Figure 4.16 Growth of isolate BF68 on TSI agar media after incubation for 24 hours at 37°C, illustrating glucose and lactose / sucrose fermentation with gas production resulting in acidic slant and acidic butt with rising in media.

4.6.8 Urease Test

The urease test was performed to detect the presence of urease enzyme in bacteria. The colour change of media from yellow to pink colour, it is indication of positive result however, no colour change indicated negative result. Out of 117 Gram negative isolates, 74(63%) isolates gave positive result for urease test while 43(37%) were gave negative result for urease (Figure 4.18).

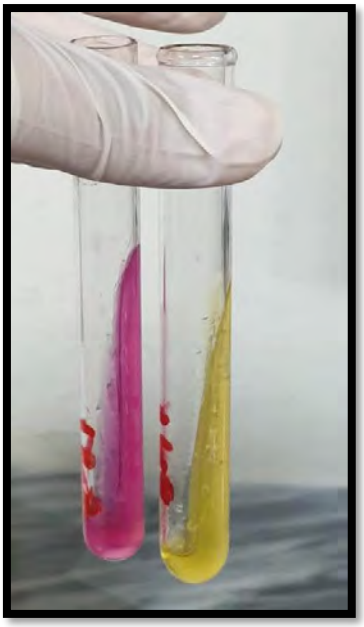

Figure 4.17 Urease test performed on Christian urea broth after incubation for 24 hours at 37°C illustrates positive result for isolate LF 167 by colour change of media from yellow to pink and negative result for isolate LF 193 by no change in colour.

4.7 Classification Using Bergey's Manual of Determinative Bacteriology for the Identification:

After performing all the biochemical tests for *Enterobacteriaceae*, results were further compared with Bergey's Manual of Determinative Bacteriology, for the identification of *Enterobacter* spp. Out of 117 Gram negative isolates, only 23 *Enterobacter* spp. were confirmed from both food and abiotic samples. Out of these 23 *Enterobacter* species, 12 were identified from food samples and 11 were identified from abiotic samples. In case of food samples, 4 *Enterobacter* spp. isolates were identified from regular MG events and 8 *Enterobacter* spp. were identified from special MG events. In case of abiotic samples, 9

Enterobacter spp. were identified from regular MG events and 2 *Enterobacter* spp. were identified from special MG events.

In current study, total 50 *Enterobacter* spp. were subjected to different phenotypic virulence assay. Among these 50 isolates, 27 *Enterobacter* spp. were previously identified from food and abiotic samples from MGs. Out of these 27 *Enterobacter* species, 17 isolates were from food samples and 10 isolates were identified from abiotic samples.

4.8 Confirmation of *Enterobacter* on Eosin Methyl Blue agar

This medium was used for the identification of coliforms and differentiating the lactose fermenters. All isolates were streaked on the EMB agar and incubated for 24 hours at 37°C. *Enterobacter* spp. give light pink to purple colour colonies on EMB agar plate. Out of 50 isolates, all isolates gave light pink to purple colour, confirming *Enterobacter* spp.

4.9 Phenotypic screening of Virulence Factors

4.9.1 Haemolysin Assay

Haemolysin production assay was performed to check the ability of *Enterobacter* for alpha haemolysin production. Complete lysis of RBCs was indicated by clear zone around the colony and were considered as β-haemolysis. α-haemolysin partially lysed RBCs which reduced haemoglobin to methaemoglobin in the medium and colour changes to green or brown. However, no change in media colour depicts gamma haemolysis i-e no haemolysis (Figure 4.19). Out of 50 *Enterobacter*, 4(8%) were beta-haemolysin producer, 2(4%) were alpha-hemolysin producer while remaining 44 (88%) had no haemolysin. A comparison of haemolysis shows that *Enterobacter* spp. differ in haemolytic ability depending upon type of sample (Figure 4.20). On comparing this virulent determinant among food and inanimate objects it was observed that *Enterobacter* spp. from food samples had higher potential for RBCs lysis as both alpha and beta hemolysin were detected whereas in abiotic samples only the beta hemolysin was detected.

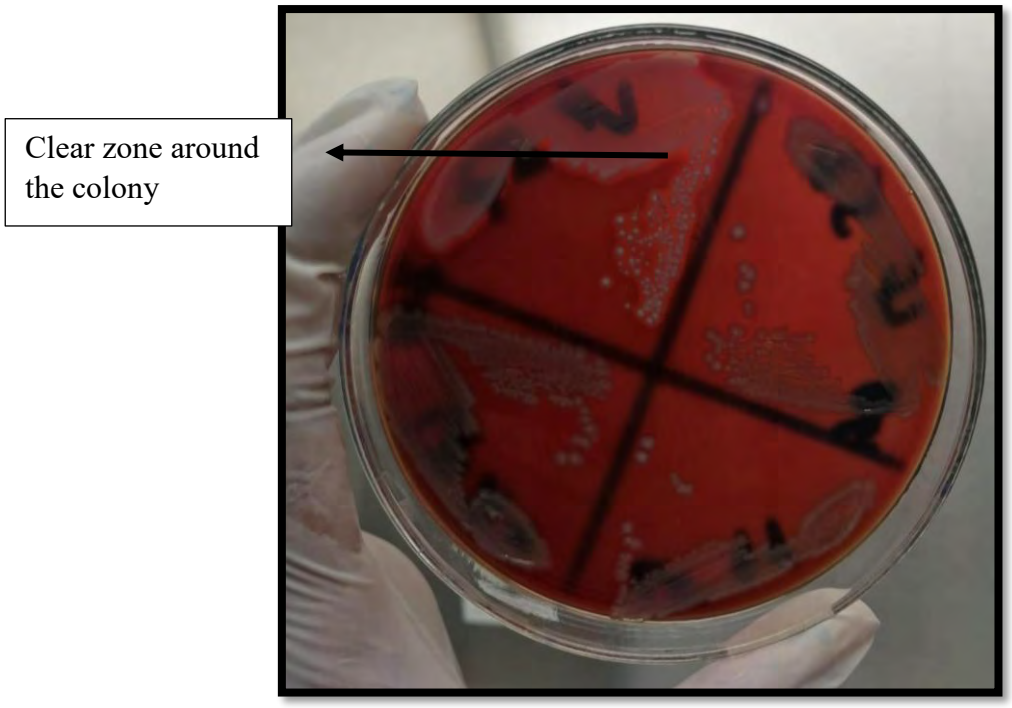

Figure 4.18 Beta hemolysin production by *Enterobacter* spp. isolate RS 380 produced a clear zone as a result of complete breakdown of RBCs and isolate RS 311, RS 329 and RS 355 produced no zone on sheep blood agar after 24 hours of incubation at 37℃.

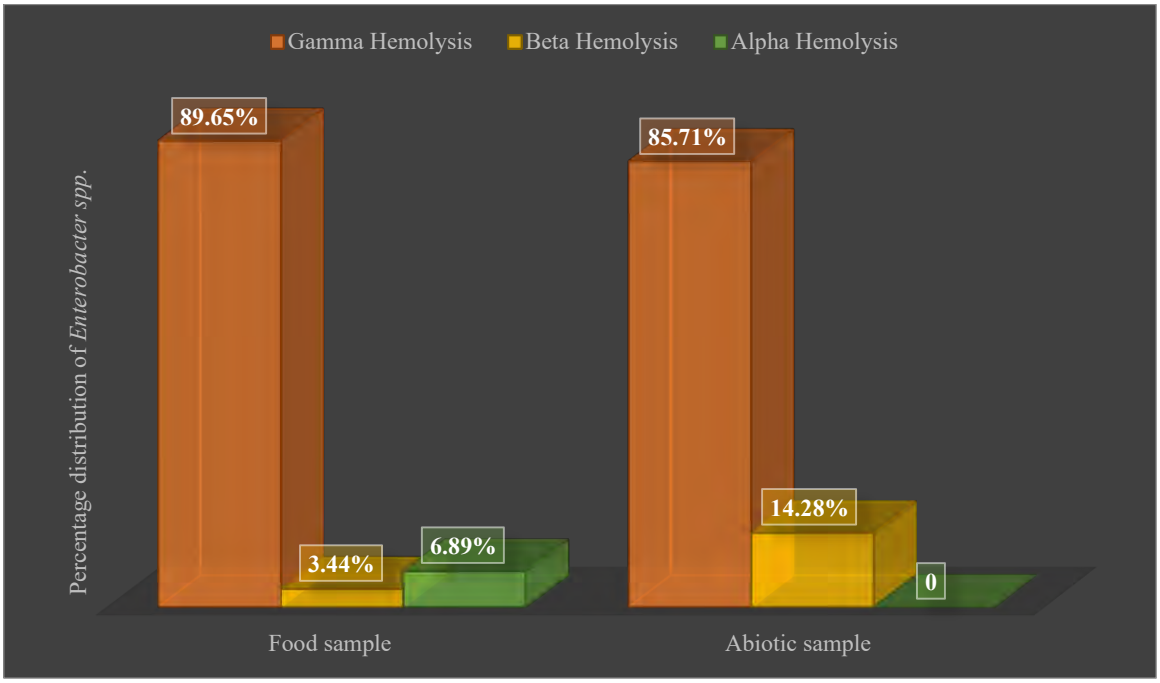

Figure 4.19: Percentage distribution of *Enterobacter* spp. among food and abiotic samples based on Hemolysin assay.

4.9.2 Biofilm Assay

Biofilm forming ability of *Enterobacter* was assessed by performing two different methods.

4.9.2.1 Congo red assay:

Congo red assay was performed on *Enterobacter* spp. to determine the biofilm forming ability, based on colony morphology on Congo red agar plate. Isolates showing red-pink colour colonies were non-biofilm formers while colonies with crystal black colour were strong biofilm formers as shown in Figure 4.21. Out 50 *Enterobacter* spp., 12(24%) were observed as strong biofilm formers, 18(36%) as moderate biofilm formers and 1(2%) as weak biofilm formers however 19(38%) were non-biofilm formers (Table 4.2).

Figure 4.20 Growth of *Enterobacter* spp. on Congo red agar to differentiate between isolate LF 125 biofilm formers (crystal black colonies), isolate LF 138 and LF 122 moderate (light black colonies) and isolate LF 133 non-biofilm formers (orange-coloured colonies) after incubation of 24 hours at 37℃.

On comparing this virulent determinant among food and inanimate objects it was observed that *Enterobacter* spp. from food samples showed highest percentage (27.58%) of strong biofilm formers by CRA method than abiotic samples (Figure 4.22).

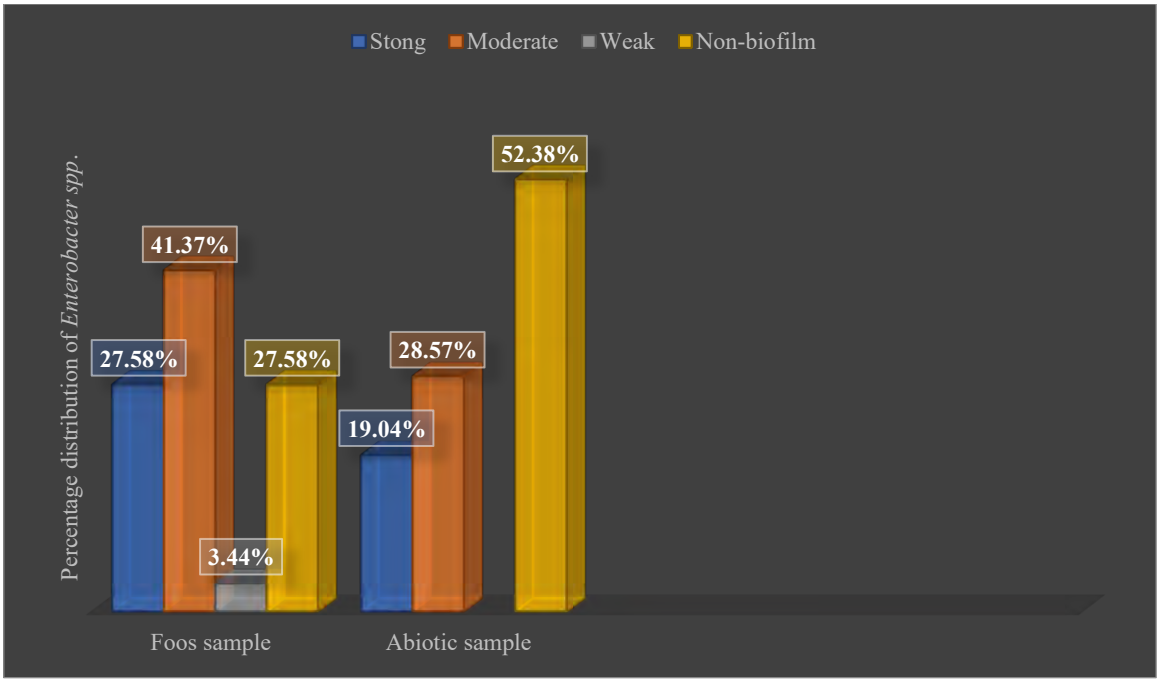

Figure 4.21 Percentage distribution of *Enterobacter* spp. based on biofilm forming ability by CRA method among food and abiotic samples.

4.9.2.2 Microtiter plate assay:

According to MTP assay, cut of OD was calculated. Strong biofilm formers were 23(46%), 7(14%) were moderate biofilm formers, 2(4%) were weak and 18(36%) non-biofilm formers from 50 *Enterobacter* spp. (Figure 4.23).

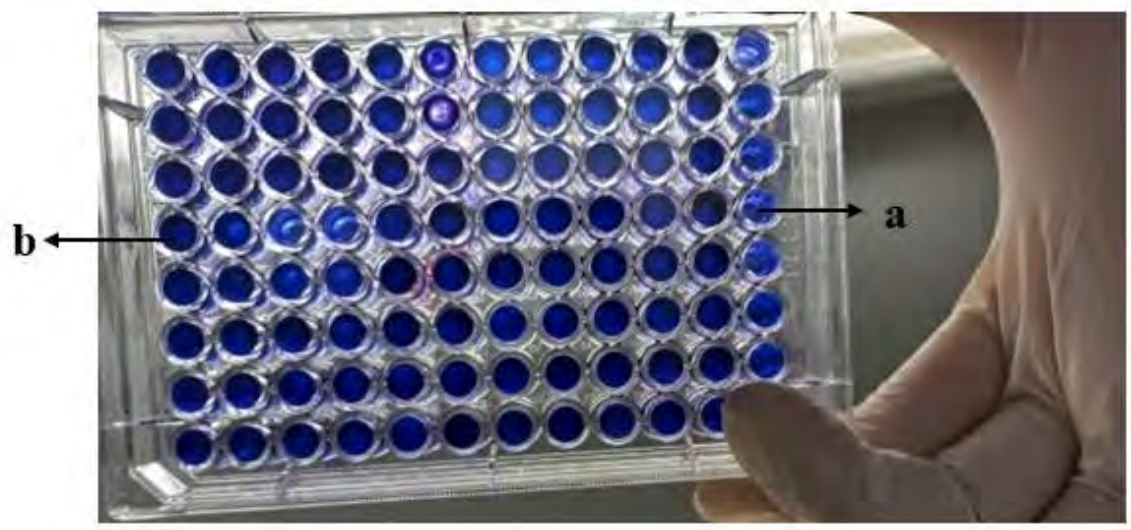

Figure 4.22 Biofilm development of *Enterobacter* spp. on microtiter plate after 24 hours of incubation at 37°C, staining with 1 % Crystal violet to differentiate (a) non-biofilm former from (b) strong biofilm former.

On comparing this virulent determinant among food and inanimate objects it was observed that *Enterobacter* spp. from food samples showed highest percentage 43.74%. of strong biofilm formers by MTP assay method than abiotic samples (Figure 4.24).

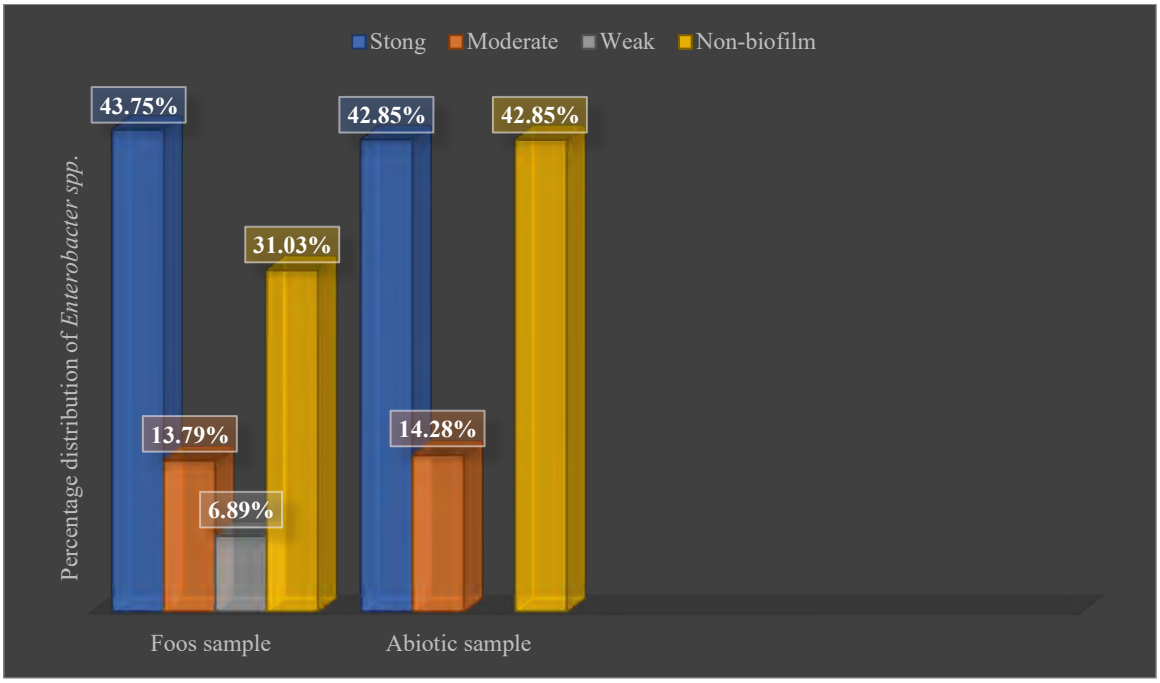

Figure 4.23 Percentage distribution of *Enterobacter* spp. based on biofilm forming ability by MTP assay among food and abiotic samples.

On comparing the quantitative and qualitative method of biofilm detection from food and abiotic samples (Table 4.4). It was observed that qualitative method (MTP assay) detects more accurately the biofilm formers as compared to quantitative method (CRA). Among food samples the highest percentage (43.74%) of strong biofilm formers were detected by MTP assay method (Figure 4.25) and from abiotic samples, the highest percentage 42.85% of strong biofilm formers were detected by MTP assay method (Figure 4.26).

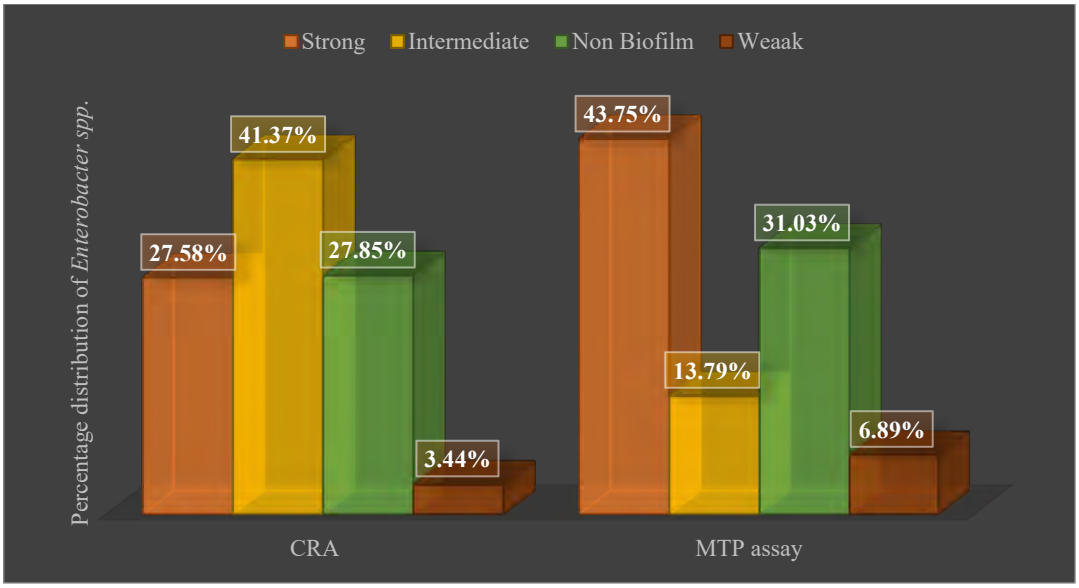

Figure 4.24 Comparative analysis of biofilm forming ability of *Enterobacter* spp. by CRA and MTP assay among food samples.

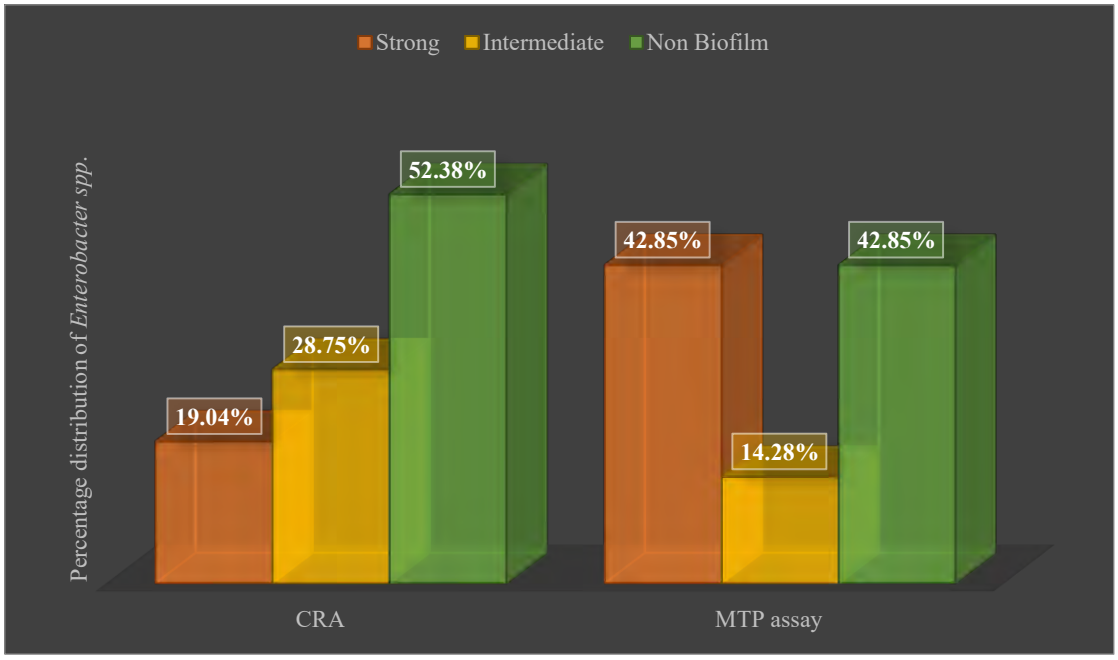

Figure 4.25 Comparative analysis of biofilm forming ability of *Enterobacter* spp. by CRA and MTP assay among abiotic samples.

4.9.3 Hemagglutination Assay

Haemagglutination assay was performed by following the protocol described by Hrv *et al*., (2016) with slight modifications. Haemagglutination activity of *Enterobacter* spp. was observed, 48(96%) agglutinated RBCs of blood group A, 43(86%) agglutinated RBCs of blood group AB whereas 14(28%) agglutinated RBCs of blood group B (Figure 4.27).

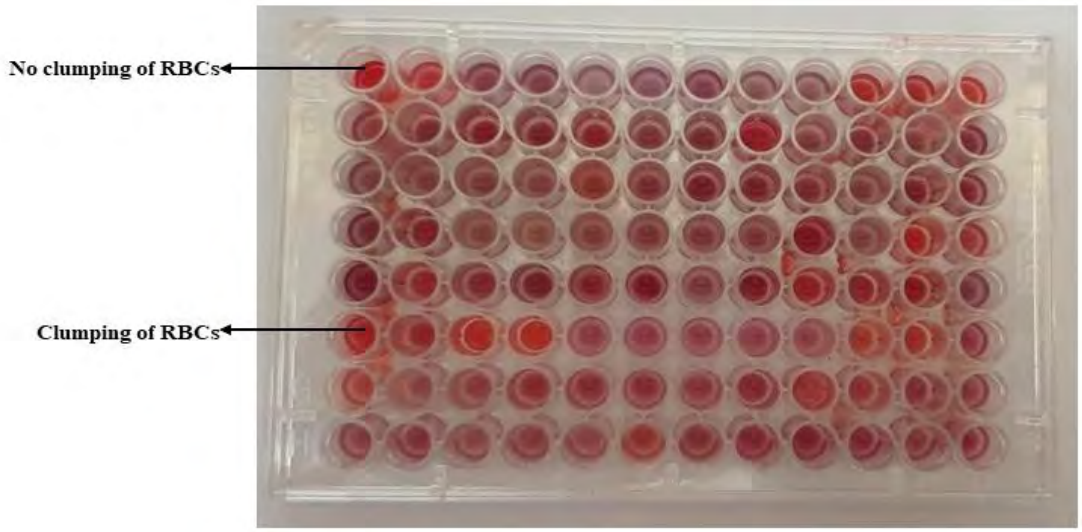

Figure 4.26 Hemagglutination activity of *Enterobacter* spp. isolates for different blood group A, AB and B after 24 hours of incubation at 37℃ illustrating clumping of RBCs positive result and no clumping of RBCs depicting negative results.

Comparative analysis of Haemagglutination assay from different blood groups was assessed separately among food and abiotic samples (Table 4.5) and it was observed that the highest percentage of clumping of RBCs was observed with blood group A.

Table 4.1 Comparison of Haemagglutination activity of *Enterobacter* spp. with blood group A, blood group B and blood group AB from food and abiotic samples

Enterobacter spp.	**Blood Group A**		**Blood Group B**		**Blood Group AB**	
	Positive n (%)	**Negative n (%)**	**Positive n (%)**	**Negative n (%)**	**Position n (%)**	**Negative n (%)**
Food	28 (96.55)	1 (3.44)	9 (31.03)	20 (68.96)	25 (91.30)	4 (8.68)
Abiotic	20 (95.23)	1 (4.76)	5 (23.80)	16 (76.19)	18 (85.71)	3 (14.28)
Total	48 (96)	2 (4)	14 (28)	36 (72)	43 (86)	7(14)

Table 4.2: Overall comparison of various virulence factors of *Enterobacter* spp. isolated from MGs of Pakistan

Virulence Factors	Scale		Food Sample n (%)	Abiotic Sample n (%)	Total n (%)
Congo	Strong BF		8 (27.58)	4 (19.04)	12 (24)
	Intermediate BF		12(41.37)	6 (28.57)	18(36)
	NBF		8(27.58)	11 (52.38)	19 (38)
	Weak BF		1 (3.44)	NIL	1 (2)
MTP	Strong BF		14 (43.75)	9 (42.85)	23 (46)
	Intermediate BF		4(13.79)	3 (14.28)	7 (14)
	NBF		9(31.03)	9 (42.85)	18 (36)
	Weak BF		2(6.89)	NIL	2 (4)
Hemolysin	Alpha hemolysis		2 (6.89)	NIL	2 (4)
	Beta hemolysis		1(3.44)	3(14.28)	4 (8)
	Gamma hemolysis		26 (89.65)	18 (85.71)	44 (88)
Hemagglutination	Blood Group A	Positive	28 (96.55)	20 (95.23)	48 (96)
		Negative	1 (3.44)	1 (4.76)	2 (4)
	Blood Group B	Positive	9 (31.03)	5 (23.80)	14 (28)
		Negative	20 (68.96)	16 (76.19)	36 (72)
	Blood Group AB	Positive	25 (91.30)	18 (85.71)	43 (86)
		Negative	4 (8.68)	3 (14.28)	7(14)

Chapter 5: Discussion

The present study was carried out to determine the virulence determinants of *Enterobacter* spp. isolated from mass gatherings of Pakistan. *Enterobacter* spp. are emerging opportunistic pathogens that could be responsible for severe infections in humans (Mishra *et al.,* 2020). WHO in 2017 had enlisted pathogens against which new antibiotics were urgently needed and designated as "priority status", *Enterobacter* spp. is one of them (De Oliveira *et al.,* 2020). Worldwide, numerous cases of foodborne illness are reported due to contaminated food by the foodborne pathogen. *Enterobacter* spp. was reported in foodborne outbreak in high school of China due to consumption of contaminated supper (Yong *et al.,* 2018). Different reports have confirmed that there is presence of microorganisms on inanimate objects in perspective of various factors like human occupancy and their activities, temperature, and humidity level (Viegas *et al,* 2022), but no study has been reported on detection of pathogenic species from any MG events from Pakistan, even very few studies globally are in literature.

The interaction between the pathogen, food, host, and objects in the environment determines the extent of foodborne illnesses and its transmission (Gourama *et al.,* 2020). Globally, multiple cases of foodborne infections were observed and reported because of food contamination by the foodborne pathogens. In several cross-sectional studies from Pakistan, prevalence of foodborne pathogens had been reported in different food items which were responsible for causing foodborne disease, leading to outbreaks (Munir *et al.,* 2019). Currently, data regarding virulence profiling of *Enterobacter* spp. from food and inanimate objects in MG events is missing from Pakistan. However, the data is available on detection of *Enterobacter* spp. from inanimate objects of hospital environment (Ejaz *et al.,* 2018). Till date, no study has been published on determination of virulence determinants of *Enterobacter* spp. in food and inanimate objects of MG events.

Different types of mass gatherings in Pakistan are organized annually, which are of various nature from religious festivals to political events. In this study, different mass gathering sites were covered from four different cities of Pakistan which include Islamabad, Sargodha, Lahore and Attock. Mass gathering events were categorised into special mass gathering events and regular mass gathering events. Special MG events sites included religious events from 3 sites (Sargodha, Lahore and Attock), cultural events from 2 sites (Islamabad and Sargodha) and food festivals only from Islamabad. Regular MGs include those that happen periodically like gatherings in various soup kitchens. However, regular MG events site included only region of Islamabad, but 3 different locations were selected which included G8 (PIMS, Sylani langarkhana), G9 (Ehsas Panah Gah) and I-11 (Ehsas Panah Gah). Such events are regular part

of life in all countries of the globe like Kum mela (India), Asian Games, Winter Olympics, Neuroz celebrations *etc.*

In the present study different hygienic practices was observed among the food handlers, majorly food handlers were not wearing any head nets or gloves, only few of them were wearing head nets but not wearing gloves. In the special mass gatherings, there was no maintenance of cleanliness and proper system of waste disposal was not available. The behavioural attributes of the attendee and staff was observed thoroughly which showed no practicing of hand washing or hand sanitizing by attendee and staff in both special and regular MG events. In both regular and special MGs, no proper cleanliness of tables, benches, chairs, and other objects was maintained, which may increase the probability of cross commination among individuals using the same object repeatedly. The unwashed hands frequently touched tables and railings in MG events increasing the chance of infections. During the survey, lack of hygienic practices was thoroughly observed at the regular MG events site. The possible reason for contamination could be regular use of same objects without proper cleaning of bench, table, tap and without proper washing of plate, glass, and jug. Also, handwashing and other hygienic practices were not observed among attendees of regular MG events. The conditions of food storage and placement on temporary stalls was also observed. In this aspect, few food handlers placed the raw and cooked food within close proximity, some food handlers placed the food items along with used disposal items which could be major source of cross contamination in food items.

The reasons for food contamination could be due to large number of attendees (more than 1000) and limited space with less SOPs for cleanliness and food storage. Also, food is prepared in bulk quantity in these special MGs events for a large number of attendees with negligence of hygienic practices. This improper preparation of food along with serving it for a whole day might increase the chances of food contamination in special MG.

In Pakistan, different studies reported the prevalence of *Enterobacter* spp. in different food items. A study conducted in Islamabad by Zeb *et al.,* 2020, found *Enterobacter* spp. (11.3%) contamination in ready to eat food (RTE) items from different venders within hospital environment. In same study, different bacterial species including *Enterobacter* spp. were isolated from abiotic samples which were implicated as possible cause of cross contamination of food items within hospital environments (Zeb *et al.,* 2020). Another study reported the prevalence *Enterobacter* spp. from food available in open market of Pakistan, it was done on

food samples like chicken samosa, potato fries and sandwich (Rakha *et al.,* 2022). Currently, in Pakistan data regarding foodborne pathogen prevalence in food items available in different mass gathering events is still missing.

In this study high percentage (52%) of *Enterobacter* spp. in food samples from different MG events in Pakistan was detected. Majorly (66%) *Enterobacter* spp. were identified from special MG events. This study also included 17 previously identified *Enterobacter* spp. isolates. In abiotic sampling, the number of *Enterobacter* spp. identified was less as compared to food samples, but majority isolates (82%) were from the abiotic samples of regular MG events. This study included 10 previously identified *Enterobacter* spp. isolates. A total of 50 *Enterobacter* spp. were analysed for phenotypic virulence assays

Virulence profiling of *Enterobacter* spp. from different MG events was performed using standard microbial techniques and protocols. Four different virulence assessments were performed which were biofilm formation assay (Congo red assay and microtiter plate assay), hemolysis assay and haemagglutination assay.

Hemolysin activity is a virulence factor which contributes to infections. Alpha haemolysis majorly play role in UTI infections and is considered as partial haemolysis however, beta hemolysin is complete haemolysis *i-e.* complete breakdown of RBCs and is involved in numerous infections (Lakna, 2018). Beta hemolysin is considered as main virulent factor for pathogen that can cause infection (Valliammai *et al*., 2020). The findings in current study show that 8% of *Enterobacter* spp. had the ability to produce beta hemolysin while 4% of *Enterobacter* spp. had the ability to produce alpha hemolysin. A study was conducted on poultry feed of chicken and *Enterobacter* spp. isolated from samples showed 99% beta hemolysin activity (Islam *et al.,* 2019). Another study conducted on *Enterobacter* spp. isolated from different environmental sources possessed haemolytic activity (both alpha and beta) (Curiel-Maciel *et al*., 2020). Study conducted by Molochaeva *et al.,* (2023), highlighted that 61.5% of *Enterobacter aerogenes* were characterized by haemolytic activity, and 42.9% *Enterobacter cloacae* showed the haemolytic activity which were isolated from different dairy product in Russia. All of the β haemolytic *Enterobacter* spp. were isolated from abiotic surface indicate that such *Enterobacter* spp. can be potentially pathogenic and be source of transmission in MGs.

Biofilm formation, a virulence factor either on biotic or abiotic surfaces is important in development of infection by bacterial species. As bacteria in biofilm are less exposed to

antibiotic treatment and can express their virulence factors more efficiently than in planktonic cells (Tula *et al.,* 2018). In the present study, *Enterobacter* spp. were screened for their biofilm forming ability. Out of 50 *Enterobacter* spp., 24% were strong biofilm formers and 38% were non-biofilm formers observed through CRA method. However, from 50 *Enterobacter* spp., 46% were strong biofilm formers and 36% were non-biofilm formers observed from MTP assay. The overall result showed that there is a high percentage detection of biofilm formers by MTP assay. This indicates that MTP assay is better assay for detection of biofilms as it is quantitative analysis. A study from Egypt, coincides with the present study as they reported the second highest prevalence of *Enterobacter* spp. from variety of food samples, where 67% of *Enterobacter* spp. were strong biofilm former using MTP assay (Edris *et al.,* 2023). On contrary, a comparative study by Mishra *et al*., (2020), highlighted that most of the clinical *Enterobacter* isolates are strong biofilm producer rather than environmental *Enterobacter* isolates. In case of present study *Enterobacter* spp., strong biofilm formers were from food samples, it may be due cross contamination during food preparation.

Furthermore, Haemagglutination assay depicts clumping of RBCs by bacteria, which is marker for identification of type 1 fimbriae among pathogenic members of *Enterobacteriaceae.* Due to presence of fimbriae, bacteria can agglutinate RBCs (Gharrah *et al.,* 2017). The pilli are one type of adhesion or virulence factor, which play important role for bacterial adhesion to a host cell mucous membrane surface, initiating infection followed by colonization. Two basic types of pilli which are short attachment pili and long conjugation pili are formed by bacterial species, it consists of hundreds of pillin protein subunits. Another hemagglutinin proteins are lectins (in bacteria involved in infections), which agglutinates the human erythrocyte with ABO blood type. Lectins interact with carbohydrates present on the surface of humans' erythrocyte like A's blood type erythrocyte surface N-Asetil-D-Galactosamine carbohydrate, B blood type's D-Galactose, and O blood type's L-fucose in specific and reversible manner to cause agglutination (Darmawati et al., 2019). The ability of *Enterobacter* species for haemagglutination was assessed in this study.

The majority of *Enterobacter* spp. showed haemagglutination with blood group A (96%) as compared to blood group AB (86%) and blood group B (28%). The different agglutination activities against human blood A, B and AB are due to the receptors on the surface of erythrocyte belonging to different blood groups. A study reported that 33.87% of *Enterobacter cloacae* isolates identified from different dairy samples had the ability to hemagglutinate RBCs (Molochaeva *et al.,* 2023). Also, Hemagglutination assay for detection of type-1 fimbriae from

a previous Indian study revealed that all environmental and clinical *Enterobacter* isolates tested positive for fimbriae (Mishra *et al.,* 2020). However, no data has particularly reported different agglutination activities against human blood A, B and AB erythrocyte. These *Enterobacter* spp. isolated in the current study depicted ability to haemagglutinate different blood group type but predominately type A is another indicator of their ability to cause infection.

Overall, in nutshell, comparative analysis of virulence determinants was done among food and abiotic samples collected from different MG events in Pakistan. From Food samples, highest percentage of biofilm forming (43.75%) *Enterobacter* spp. was observed. Majorly (52%) of these biofilms forming *Enterobacter* spp. of food were isolated from special MG events rest biofilm forming *Enterobacter* spp. were isolated from regular MG events held in Islamabad. From abiotic sample, highest percentage of biofilm forming (42.85%) *Enterobacter* spp. were identified from regular MG events. In the current study, association of biofilm formers with clumping of RBCs was also analysed. From MTP assay, highest percentage of biofilm forming (43.75%) *Enterobacter* species had 100% association with blood group A, then 78% with blood group AB and least 35% with blood group B. Type 1 fimbriae play role in the first step of biofilm formation by enhancing bacterial attachment to mucosal epithelium. Also, Haemagglutination is mediated by Type 1 fimbriae, which depicts a correlation between haemagglutination and biofilm formation. A similar study had revealed that fimbriae contribute to the virulence and Type 3 fimbriae aid in biofilm formation in uropathogenic *E.coli* (Adenipekun EO *et al.,* 2018).

Overall, there was a high prevalence of *Enterobacter* spp. in mass gathering, which were significantly virulent except for expression of hypermucoviscosity. The present study provide data as no previous data regarding virulence profiling of *Enterobacter* spp. from MG events is available and if such potential spread in community setting from MG events , it will have grave health issue in Pakistan which need serious attention by public health authorities.

Chapter 6: Conclusions

The main findings of the study are:

1. High prevalence of *Enterobacter* spp. found in the food samples compared to abiotic samples.
2. Comparatively *Enterobacter* spp. were more isolated from special MGs.
3. From the special MGs, more *Enterobacter* spp. were identified especially from food samples.
4. From regular MGs, more *Enterobacter* spp. were identified from abiotic samples.
5. Potential pathogenic strains as combination of tested virulence factors were present in isolated *Enterobacter* that shows that there is persistence in host environment.
6. Pathogenic *Enterobacter* strains were more prevalent in food samples as compared to abiotic samples except for the haemolysin determinant.

chapter 7: Future Prospects

The study was carried out to determine the virulence factors of *Enterobacter* spp. isolated from different MGs of Pakistan. However, further studies on virulence factors are needed to highlight its role in different foodborne diseases and epidemics.

- There must be awareness programs to educate people regarding personal and public hygiene practices to contain spread of infections in the mass gatherings.
- Genotypic detection of food borne *Enterobacter* virulence genes should be studied.
- There is need to study correlation between antibiotic resistance and virulence factors for better understanding of pathogenicity of *Enterobacter* spp.
- Evidence based preventive measures should be taken to ensure food safety.

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Appendix-I

Table A-I: Essential Components of Nutrient Agar Medium (Oxoid® - Nutrient Agar CM0003)

Typical Formula	**g/L**
`Lab-Lemco' Powder	1.0
Yeast Extract	2.0
Peptone	5.0
Sodium Chloride	5.0
Agar	15.0
pH 7.4 ± 0.2 at 25°C	

Table A-II: Essential Components of MacConkey Agar Medium (Sigma-Aldrich® - MacConkey Agar 70415)

Typical Formula	**g/L**
Bile Salts	5.0
Neutral Red	0.075
Lactose	10.0
Peptone	20.0
Sodium Chloride	5.0
Agar	12.0
pH 7.4 ± 0.2 at 25°C	

Table A-III: Essential Components of Sim Agar Medium (Oxoid® - Sim Medium CM0435)

Typical Formula	**g/L**
Tryptone	20.0
Peptone	6.1
Ferrous Ammonium Sulphate	0.2
Sodium Thiosulphate	0.2
Agar	3.5
pH 7.3 ± 0.2 at 25°C	

Table A-IV: Essential Components of Simmons citrate Agar Medium (Liofilchem® - Simmons citrate Agar ISO 610046)

Typical Formula	**g/L**
Magnesium Sulphate	0.2
Dipotassium Phosphate	1.6
Ammonium Dihydrogen Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Brom Thymol Blue	0.08
Agar-Agar	14.0
pH 6.8 ± 0.2 at 25°C	

Table A-V: Essential Components of MR-VP Broth Medium (Oxoid® - MR-VP Broth CM0043)

Typical Formula	**g/L**
Peptone	7.0
Glucose	5.0
Phosphate Buffer	5.0
pH 6.9 ± 0.2	

Table A-VI: Essential Components of Triple Sugar Iron Agar Medium (Liofilchem® - Triple Sugar Iron Agar ISO 610350)

Typical Formula	**g/L**
Meat Extract	3.0
Yeast Extract	3.0
Lactose	10.0
Peptone	20.0
Sodium Chloride	5.0
Glucose	1.0
Sodium Thiosulfate	0.3
Ferric Ammonium Citrate	0.3
Phenol Red	0.024
Agar	12.0
pH 7.4 ± 0.2 at 25°C	

Table A-VII: Essential Components of Urea broth Medium (Liofilchem® - Urea broth ISO 610311)

Typical Formula	**g/L**
Potassium Phosphate Monobasic	10.0
Tryptone	30.0
Phenol Red	4.0mg
Sodium Chloride	5.0
Urea	20
pH 6.8 ± 0.2 at 25°C	

Table A-VIII: Essential Components of Eosin Methylene Blue Agar (Modified) Levine Medium (Oxoid® - Eosin Methylene Blue Agar Levine CM0069)

Typical Formula	**g/L**
Peptone	10.0
Lactose	10.0
Dipotassium Hydrogen Phosphate	2.0
Eosin Y	0.4
Methylene Blue	0.065
Agar	15.0
pH 6.8 ± 0.2	

Table A-IX: Essential Components of Blood Agar Base Medium (Liofilchem® - Blood Agar Base ISO 610005)

Typical Formula	**g/L**
Tryptose	10.0
Meat Extract	10.0
Sodium Chloride	5.0
Agar-Agar	15.0
pH 7.3 ± 0.2 at 25°C	

Table A-X: Essential Components of Brain Heart Infusion Broth Medium (Oxoid® - Brain Heart Infusion Broth CM1135)

Typical Formula	**g/L**
Brain Infusion Solids	12.5
Beef Heart Infusion Solids	5.0
Proteose Peptone	10.0
Glucose	2.0
Sodium Chloride	5.0
Disodium Phosphate	2.5
pH 7.4 ± 0.2 @ 25°C	

Table A-XI: Chemical composition and cell density of different concentration of McFarland turbidity standards

		Concentrations of McFarland turbidity standard				
		0.5	**1**	**2**	**3**	**4**
Chemical composition	1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
	1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1×1^8 CFU/ml)		1.5	3	6	9	12

Table A-XII: Overall Analysis of Hemolysis assay performed on *Enterobacter* isolates from Mass Gatherings

Enterobacter spp.	**Alpha Hemolysis n (%)**	**Beta Hemolysis n (%)**	**Gamma hemolysis n (%)**
Food	2 (6.89)	1(3.44)	26 (89.65)
Abiotic	-	3(14.28)	18 (85.71)
Total	2 (4)	4 (8)	44 (88)

Table A-XIII: Overall Assessment of CRA performed on *Enterobacter* from Mass Gatherings

Enterobacter spp.	**Strong n (%)**	**Intermediate n (%)**	**Weak n (%)**	**Non-biofilm n (%)**
Food	8 (27.58)	12(41.37)	1 (3.44)	8(27.58)
abiotic	4 (19.04)	6 (28.57)	-	11 (52.38)
Total	12 (24)	18(36)	1 (3.44)	19 (38)

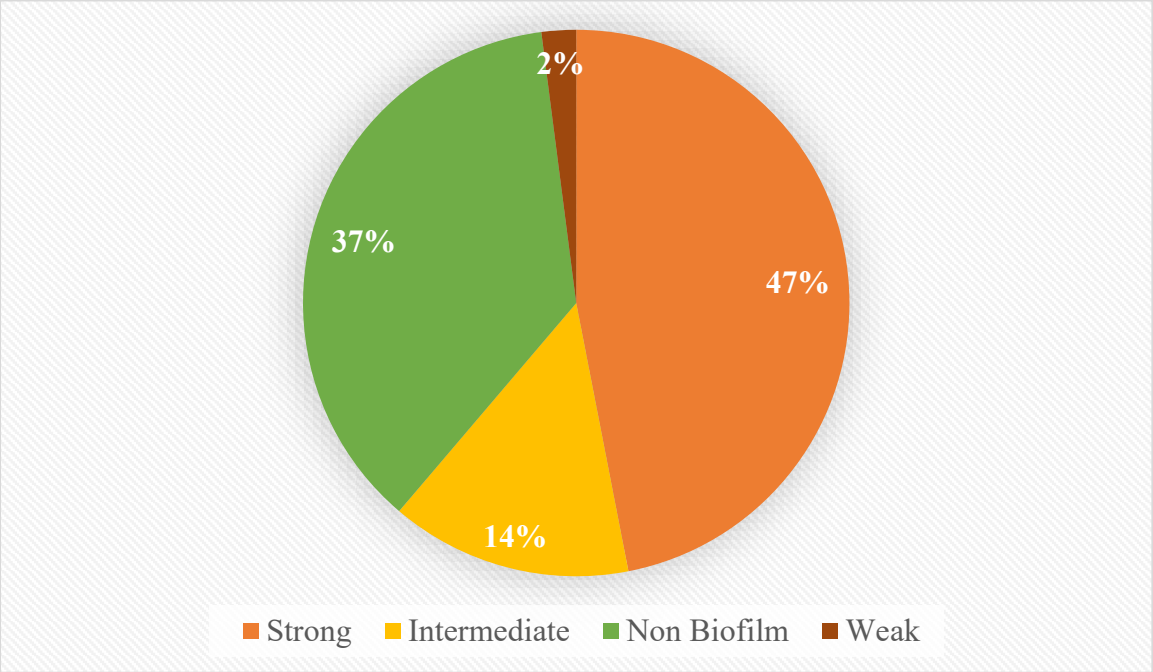


Figure A-I: Percentage Distribution of total *Enterobacter* isolates by biofilm CRA

Table A-XIV: Overall Assessment of MTP assay performed on *Enterobacter* from Mass Gatherings

Enterobacter spp.	**Biofilm Formers**			**Non-biofilm Formers n (%)**
	Strong n (%)	**Intermediate n (%)**	**Weak n (%)**	
Food	14 (43.75)	4(13.79)	2(6.89)	9(31.03)
Abiotic	9 (42.85)	3 (14.28)		9 (42.85)
Total	23 (46)	7 (14)	2(6.89)	18 (36)

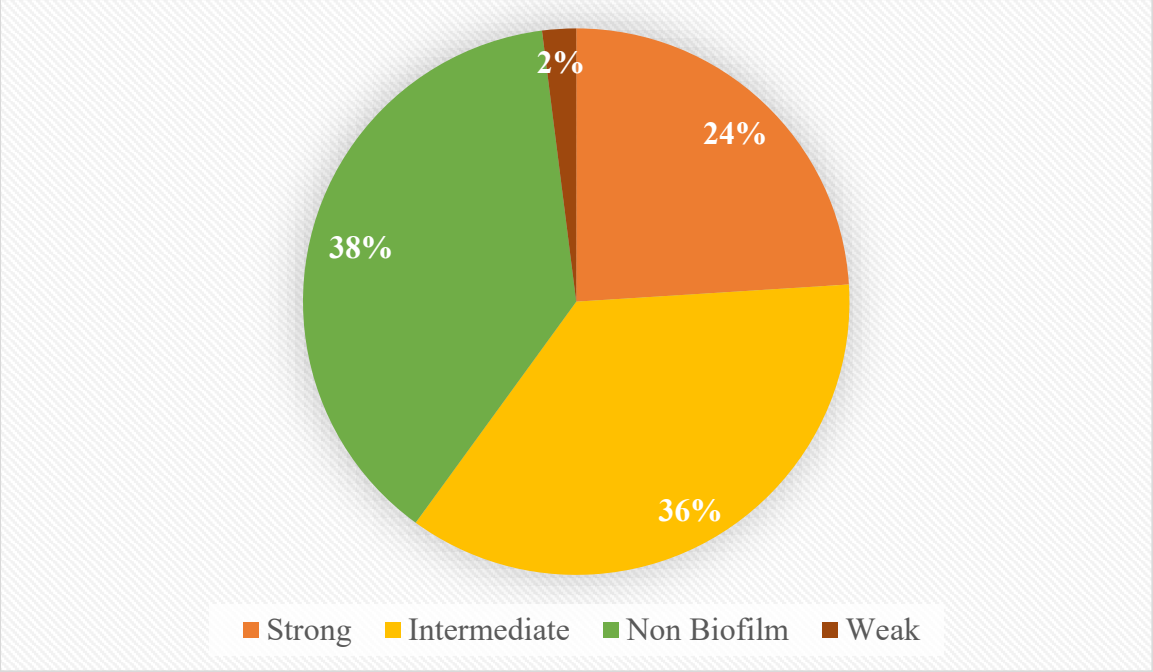


Figure A-II: Percentage Distribution of total *Enterobacter* isolates by biofilm MTP assay

Table A-XV: Comparison of CRA and MTP assay performed on *Enterobacter spp* from MGs.

Enterobacter spp.	**CRA**				**MTP**			
	Strong n (%)	**Intermediate n (%)**	**Non-Biofilm n (%)**	**Weak n (%)**	**Strong n (%)**	**Intermediate n (%)**	**Non-Biofilm n (%)**	**Weak n (%)**
Food	8(27.58)	12(41.37)	8(27.5)	1(3.44)	14(43.75)	4(13.79)	9(31.03)	2(6.89)
abiotic	4(19.04)	6 (28.57)	11 (52.38)		9(42.85)	3 (14.28)	9 (42.85)	
Total	12 (24)	18(36)	19 (38)	1 (2)	23 (46)	7 (14)	18 (36)	2 (4)

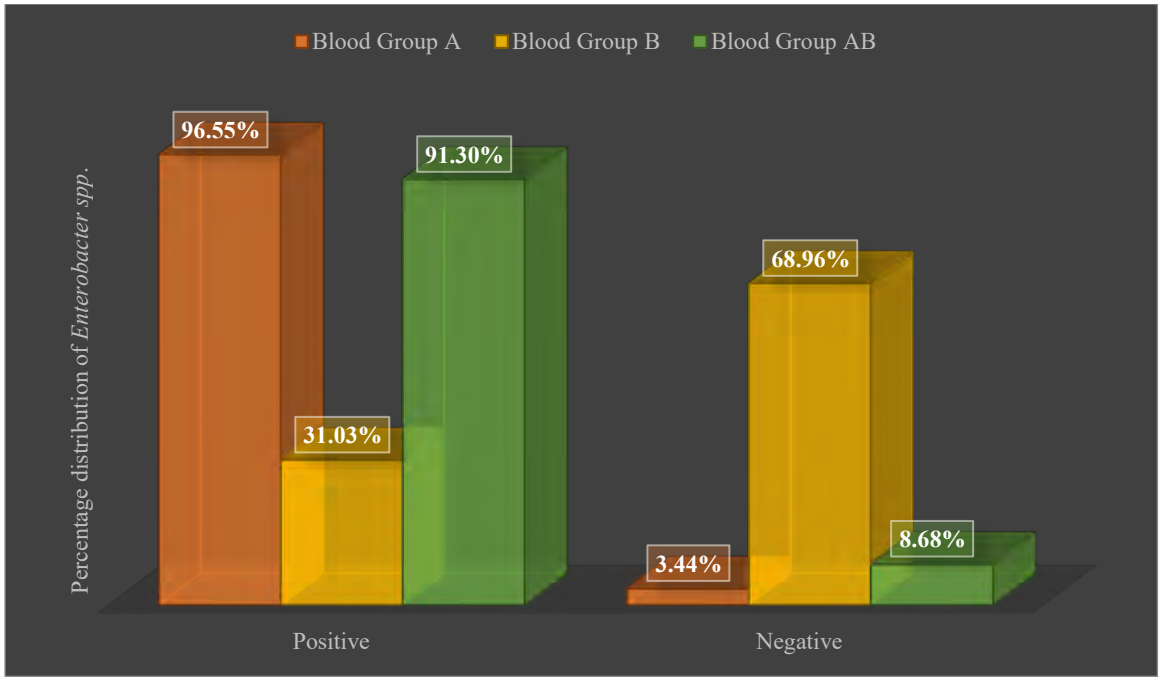


Figure A-III: Percentage distribution of *Enterobacter spp.* based on hemagglutination activity among food samples.

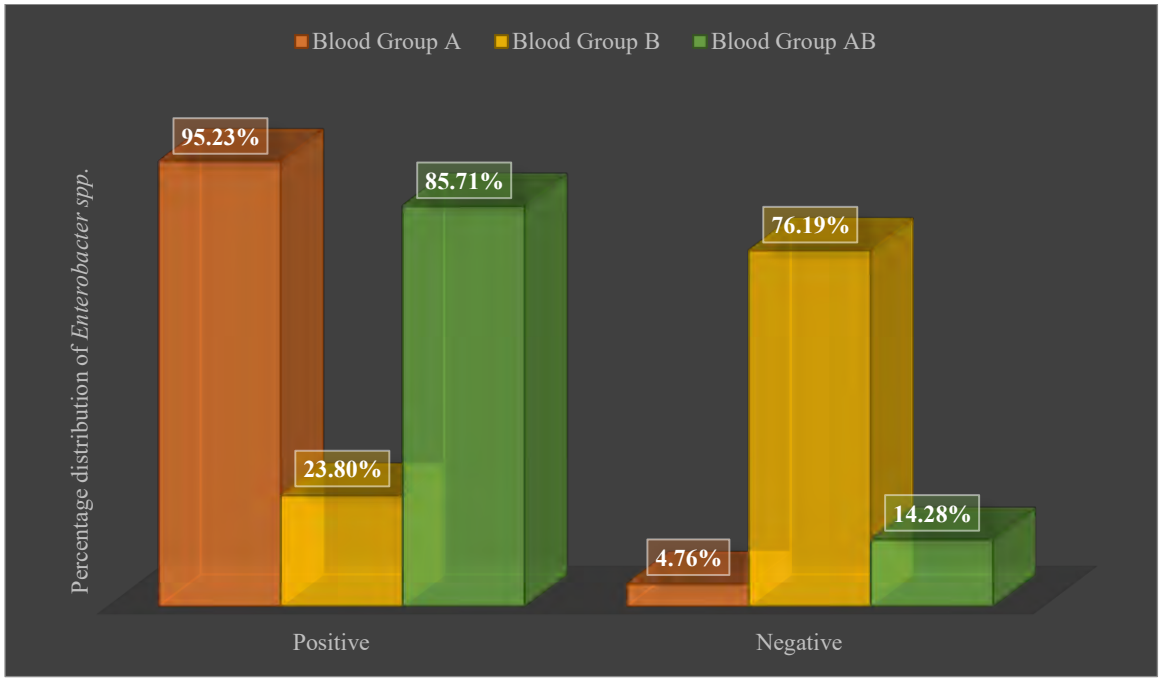


Figure A-IV: Percentage distribution of *Enterobacter spp.* based on hemagglutination activity among abiotic samples.

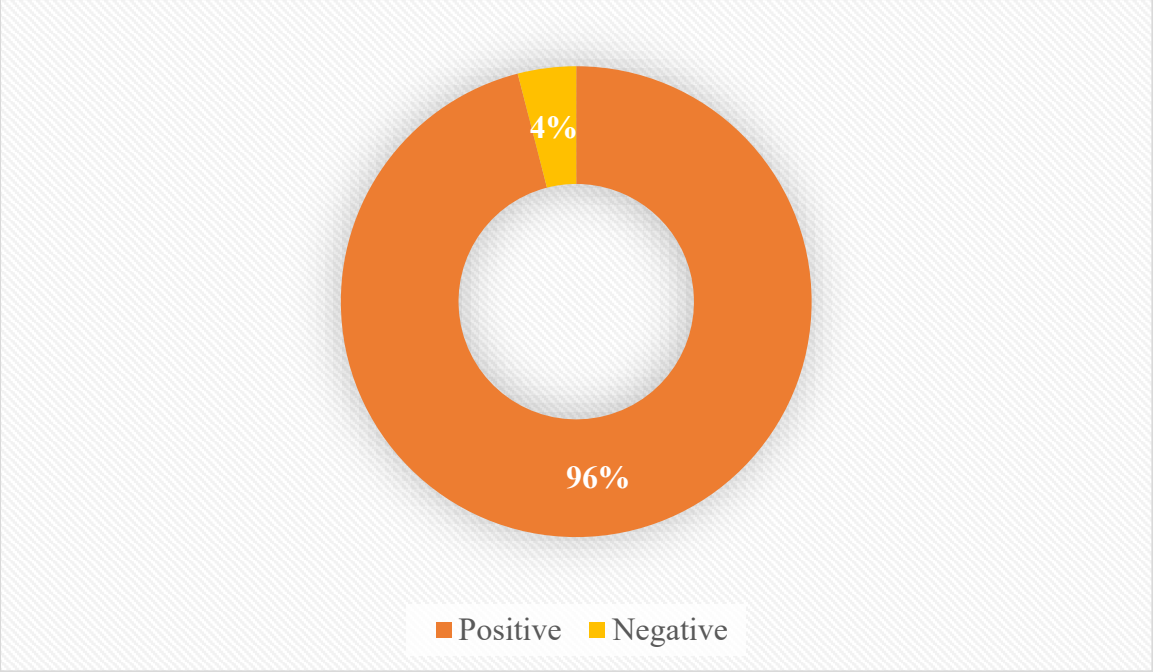


Figure Ä-V: Percentage distribution of total *Enterobacter* isolates based on hemagglutination assay (by Blood Group A) isolated from different mass gatherings of Pakistan.

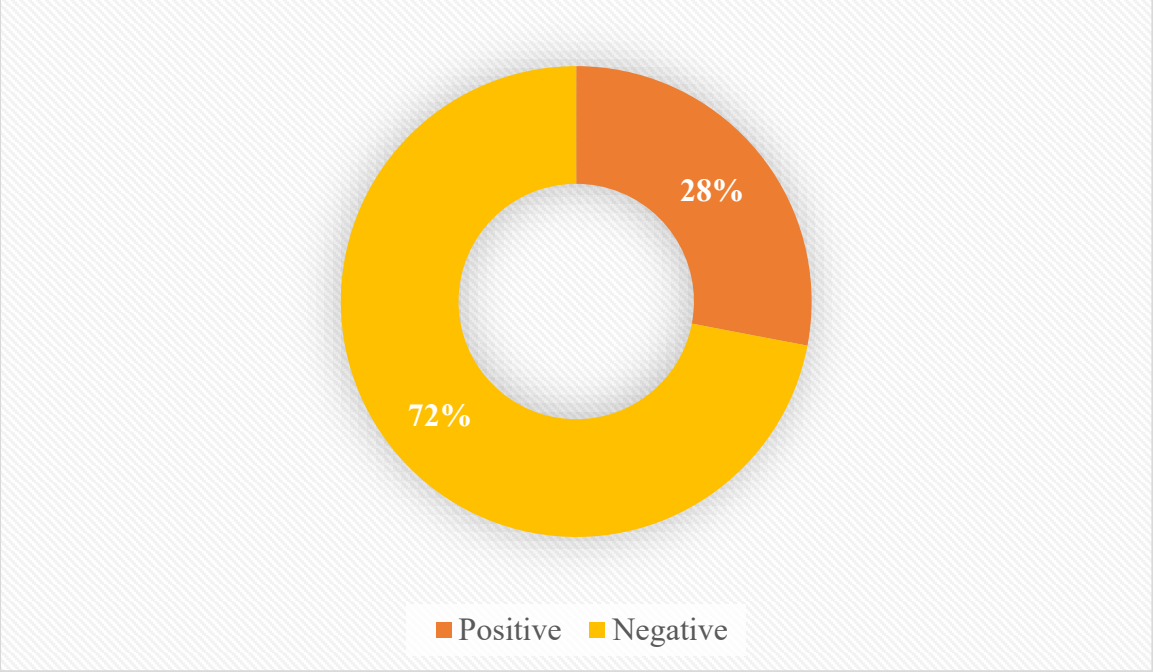


Figure A-VI: Percentage distribution of total *Enterobacter* isolates based on hemagglutination assay (by Blood Group B) isolated from different mass gatherings of Pakistan.

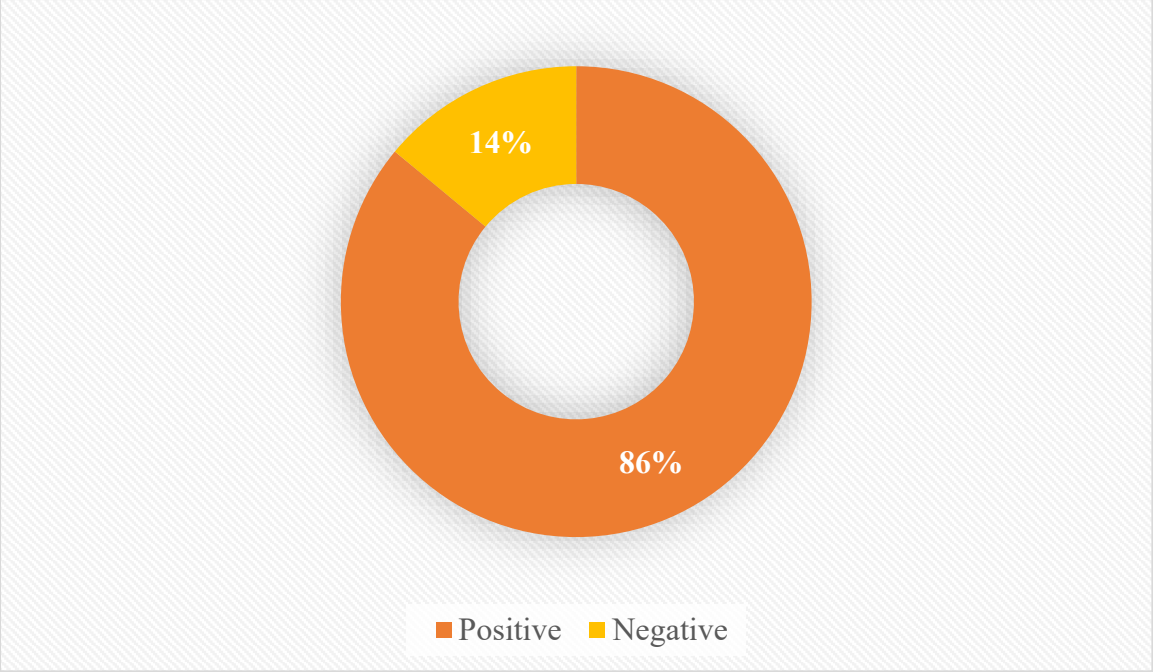
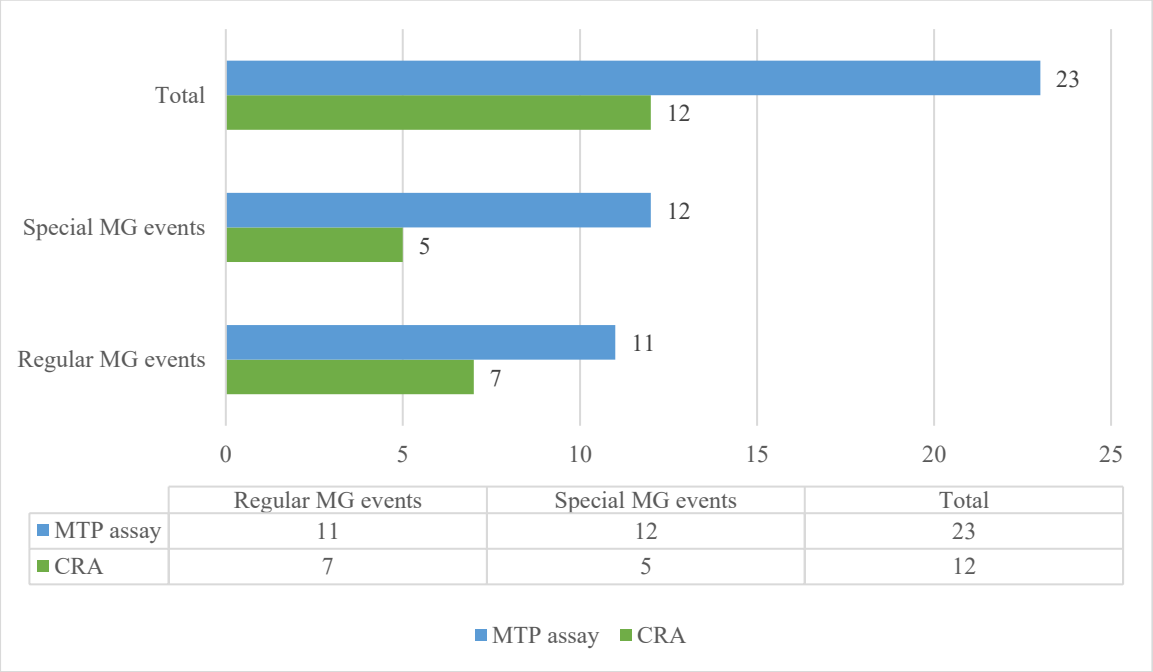


Figure A-VII: Percentage distribution of total *Enterobacter* isolates based on hemagglutination assay (by Blood Group AB) isolated from different mass gatherings of Pakistan.



	Regular MG events	Special MG events	Total
MTP assay	11	12	23
CRA	7	5	12

Figure A-VIII: Distribution of total biofilm forming *Enterobacter species* by CRA and MTP assay from different mass gatherings of Pakistan.

Table A-XVI: Association of biofilm with Haemagglutination activity performed with different blood groups on *Enterobacter spp.*

Biofilm Assay	Isolates	Blood Group A		Blood Group B		Blood Group AB	
		Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Position n (%)	Negative n (%)
CRA	Food	7 (87.5)	1(12.5)	4(50)	4(50)	7(87.5)	1(12.5)
	Abiotic	4(100)		1(25)	3(75)	3(75)	1(25)
Total		11(92)	1(8)	5(42)	7(58)	10(83)	2(17)
MTP assay	Food	14(100)		5(36)	9(64)	11(79)	3(21)
	Abiotic	9(100)		3(33)	6(67)	7(78)	2(22)
Total		23(100)		8(35)	15(65)	18(78)	5(22)

Table A-XVII Association of Hemolysis with Haemagglutination activity performed with different blood groups on *Enterobacter spp.*

Enterobacter spp.	**Beta hemolysin**	**Blood group A**	**Blood Group AB**	**Blood Group B**
Food	1 (25%)	-	1 (25%)	1 (25%)
abiotic	3 (75%)	3 (75%)	3 (75%)	-
Total	4	75% (Positive)	100% (Positive)	25% (Positive)

Appendix-II

Questionnaire

Quaid-i-Azam University Islamabad, Department of Microbiology

Prevalence and Antibiogram of Food Borne Pathogen Isolated from Mass Gathering in Twin Cities, Pakistan

The questionnaire is to study the prevalence and antibiogram of foodborne pathogen isolated from mass gathering in twin cities of Pakistan.

Name of event: ---------- **Location:** ----------

1. Type of event

a. Indoor ☐ **b**. Outdoor ☐

2. Time duration of event

a. 1 day ☐ **b.** 2 days ☐

c. 3 days ☐ **d.** Others ☐

3. Level of security

a. High ☐ **b.** Moderate ☐

c. Low ☐ **d.** No ☐

4. Nationality of attendees

a. Pakistani ☐ **b.** Foreigners ☐

5. Gender Percentage

a. Male ☐ **b.** Female ☐

6. Attendees type

a. Individuals ☐ **b.** Group of friend ☐ **c.** Families ☐

7. Estimated number of attendees

a. 100-500 ☐ **b.** 500-1000 ☐ **c.** >1000 ☐

8. Do attendees practice hygiene measure?

a. Yes ☐ **b.** No ☐

9. Stay duration of attendees

a. 30 mins- 1 hour ☐ **b.** 1 hour- 2 hour ☐

c. 2 hour- 3 hours ☐ **d.** More than 3 hours ☐

10. Cleanliness level in the event

a. Good ☐ **b**. Average ☐ **c**. Poor ☐

11. System of waste disposal

a. In waste bins ☐ **b.** In open air ☐

12. Hand washing facility availability

a. Yes ☐ **b.** No ☐

13. Do foodhandler practice hand washing?

a. Yes ☐ **b.** No ☐

14. Is food handler wearing gloves?

a. Yes ☐ **b.** No ☐

15. Is food handler wearing net cap?

a. Yes ☐ **b.** No ☐

16. Is food handlers wearing apron?

a. Yes ☐ **b.** No ☐

17. Placement of food stalls

a. Raw and cooked food stalls were separate ☐

b. Raw and cooked food stalls were not separate ☐

18. Storage of food

a. In refrigerators ☐ **b.** Placed on bench top ☐

19. Is food covered?

a. Yes ☐ **b.** No ☐

20. Is washroom facility available?

a. Yes ☐ **b**. No ☐

21. Is the washroom kept clean?

a. Yes ☐ **b**. No ☐

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