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THE EFFECT OF RIFAMPICIN AND GENTAMICIN ON TOXIN RELEASE AND THE SOS RESPONSE IN *ESCHERICHIA COLI* O104:H4

by
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Title: The effect of rifampicin and gentamicin on toxin release and the SOS response in *Escherichia coli* O104:H4

Background: *Escherichia coli* O104:H4, a new pathotype Enteroaggregative Hemorrhagic *Escherichia coli* (EAHEC), was the cause of a 2011 severe outbreak affecting European countries especially Germany. Its high virulence and novel pathophysiology seem to be due to the acquisition of a Shiga toxin 2 (Stx2) encoding prophage by horizontal gene transfer. Treatment of Shiga toxin producing *E.coli* (STEC) with antimicrobial agents is currently refrained from since it may lead to bacterial lysis, enhanced release of toxins, and exacerbation of the disease. To assess whether antimicrobial agents can be used in the treatment of the outbreak *Escherichia coli* O104:H4 strain infections, the effects of rifampicin and gentamicin were evaluated at the levels of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). They were also evaluated at two sub MIC levels to assess the SOS response, a bacterial DNA damage response, and its effect on toxin release

Methods: The MIC and MBC of rifampicin and gentamicin for the novel pathotype outbreak strain from Germany (D3774/C22711) were determined using the broth dilution method. Pulsed field gel electrophoresis (PFGE) was carried out on the new *E. coli* O104:H4 pathotype and two pre-outbreak *E. coli* O104:H4 strains (2009 EL-2050 and 2009 EL-2071 from the Republic of Georgia), to determine their genomic relatedness. Polymerase Chain reaction (PCR) analysis of the *stx2* gene in the outbreak and pre-outbreak strains was performed. The transcription level of the *stx2* gene was assessed in the 3 strains using Real time polymerase chain reaction (RT-qPCR). In addition, the transcription levels of the *stx2* and *recA* (SOS response inducer) genes were assessed using RT-qPCR in the outbreak *E.coli* O104:H4 samples treated with different regimens of rifampicin and gentamicin. Subsequently, Reverse Passive Latex Agglutination (RPLA) was used to determine the Stx2 titers in supernatants of bacterial cultures subjected to different doses and combinations of the antimicrobial agents. To examine LexA levels (SOS response repressor) in the outbreak *E.coli* O104:H4 sample subjected to different doses of antimicrobial agents, western blot was carried out. Finally, the efficacy of treatment with antimicrobial agents was assessed by infecting BALB/c mice with *E. coli* O104:H4 and then administering different combinations of rifampicin and gentamicin.

Results: The MIC and MBC of rifampicin were 16 µg/ml and 32 µg/ml respectively. On the other hand, the MIC and MBC of gentamicin were 1 µg/ml and 4 µg/ml respectively. PFGE analysis demonstrated slight genomic differences between the new pathotype and the pre-outbreak strains, denoting that these strains could be related. The stx2 gene was identified in the outbreak and pre-outbreak strains. The transcription level of the *stx2* gene in the new pathotype was 1.41 and 1.75 fold that of the 2009 EL-2050 and 2009 EL-2071

pre-outbreak strains respectively. Moreover, the transcription level of the *stx2* gene in the new pathotype, was substantially decreased as a result of treatment with the different concentrations of the antimicrobial agents, but was enhanced when the antibiotics were administered at two sub inhibitory levels. The results of the RPLA were in accordance with the RT-qPCR. Gentamicin at both sub MIC levels resulted in high transcription levels of the *recA* gene and lack of expression of the LexA protein, indicating that the SOS response was activated. Rifampicin resulted in low transcription levels of the *recA* gene at both sub MIC levels. However, it led to a high LexA protein expression at the sub MIC 2 level (four fold dilution of MIC) indicating that the SOS response was not activated, and a low expression at the sub MIC 1 level (two fold dilution of MIC). *In vivo*, the highest survival rate in BALB/c mice was observed in the group that received the MBC dose of gentamicin.
Conclusion: The Germany outbreak strain appears to be related to the pre-outbreak strains. The use of antimicrobial agents in *E. coli* O104:H4 infection seems to be effective and provides a promising ground for the treatment of human infections with this agent

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ABBREVIATIONS

A/E	Attaching and effacing
AAF	Aggregative Adherence Fimbriae
Aap	antiaggregation protein
Aai	AggR activatied island
BSA	Bovine serum albumin
CDC	Center of Disease Control and Prevention
CFU	Colony Forming Unit
CLB	Cell lysis buffer
CLSI	Clinical and Laboratory Standards Institute
CSB	Cell suspension buffer
CTD	Carboxyl Terminal Domain
CT-SMAC	Cefixime Tellurite Sorbitol MacConkey agar
dsDNA	Double stranded DNA
EAEC	Enteroaggregative *E.coli*
EAHEC	Enteroaggregative Hemorrhagic *E.coli*
EAST-1	*Escherichia coli* heat-stable enterotoxin 1
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA/EIA	Enzyme Linked Immunosorbent Assay/Enzyme Immunoassay
Elongation Factor	Elongation factor
EPEC	Enteropathogenic *E.coli*
ER	Endoplasmic Reticulum
ESBL	Extended spectrum Beta Lactamase
ETEC	Enterotoxinogenic *E.coli*
EU-RL	European union reference laboratory
Gb3/Gb4	Globotriaosylceramide/ Globotetraosylceramide
Gen	Gentamicin
GN	Gram Negative
HUS	Hemolytic uremic syndrome
IACUC	The Institutional Animal Care and Use Committee
IL	Interleukin
LB	Luria Bertani
LT	Heat liable toxin
LEE	Locus of Enterocyte Effacement
LPF	long polar fimbriae

LPS	Lipopolysaccharide
MH II	Mueller Hinton II
MIC	Minimum Inhibitory Concentration
MLST	Multilocus sequence typing
MMS	Methyl methanesulphate
MW	Molecular weight
NTD	Amino Terminal Domain
PAI	Pathogenicity island
PCR	Polymerase Chain Reaction
Pet	Plasmid encoded toxin
PFGE	Pulsed Field Gel Electrophoresis
Pic	Protein involved in colonization
Pol	Polymerase
OD	Optical density
pO157	Plasmid O157
pAA	Plasmid Aggregative Adherence
Rif	Rifampicin
RKI	Robert Koch Institute
RPLA	Reverse Passive Latex Agglutination
rRNA	Ribosomal RNA
RT-qPCR	Real-time Polymerase Chain Reaction
Set	*Shigella* enterotoxin
Sep A	*Shigella* extracellular protein A
Sig A	*Shigella* IgA protease-like homologue
SLT	Shiga-Like Toxin
SMAC	Sorbitol MacConkey
SPATES	Serine Proteases Autotransporter of *Enterobacteriaceae*
ssDNA	Single-Stranded DNA
ST (a or b)	Heat stable toxin
STEC	Shiga toxin–producing E. coli
Sub MIC	Sub-Inhibitory Concentration
TBE	Tris-Boric acid-EDTA
TBX	Trytone Bile X-Glucuronide
TE	Tris-EDTA
TGN	Trans-Golgi network
TLR4	Toll-like-receptor-4
TNF-α	Tumor necrosis factor-α
tRNA	Transfer RNA
TTP	Thrombotic Thrombocytopenic Purpura
UV	Ultraviolet
VBNC	Viable but non-culturable state

CHAPTER I

INTRODUCTION

In May 2011, one of the most severe outbreaks of *Escherichia coli* (*E.coli*) occurred in 16 countries, with cases predominantly reported in Germany. The outbreak was associated with bloody diarrhea, hemorrhagic colitis, and an unprecedented rate of hemolytic uremic syndrome (HUS) which is a fatal disease. The outbreak was contained in July 2011, after the implementation of control measures. The causative agent was identified as Shiga toxin producing *E.coli* O104:H4. The virulence of this strain arises from its novel hybrid pathotype that exhibits Enteroaggregative *E.coli* characteristics and Enterohemorrhagic *E.coli* (EHEC) ability of producing Shiga Toxin 2 (Stx2) encoded by the *stx2* gene. Prior to the outbreak, strains of *E.coli* O104:H4 that harbored the *stx2* gene have been rarely reported.

The treatment of most Shiga toxin producing *E.coli* (STEC) infections mainly focuses on supportive therapy and avoidance of antibiotics administration. The use of antimicrobial agents in the treatment of such infections might exacerbate the disease by causing lysis of bacterial cells and subsequently resulting in enhanced release of Shiga toxins (Stxs) that might have been stored in the bacteria. In addition, the increased release of Stxs might also be related to the activation of a DNA repair system in *E.coli* known as the SOS response, which reduces the efficiency of treatment.

Earlier studies conducted in the Department of Experimental Pathology, Immunology and Microbiology on EHEC O157:H7 strains indicated that using the minimal inhibitory concentration (MIC) of rifampicin that inhibits toxin expression, prior to

administration of a bactericidal antibiotic, gentamicin, at the minimal bactericidal concentration (MBC), can be an approach for treating EHEC infections, and showed that rifampicin and gentamicin at their sub-inhibitory concentrations (sub MICs) did not activate the SOS response in *E.coli* O157:H7, denoting that these antimicrobial agents are efficient for treatment purposes.

Since the outbreak *E.coli* O104:H4 strain harbors unique characteristics, potential modes of treatment, using antimicrobial agents at different doses and various combinations were determined. The aims of the study were to assess:

1) The genomic relatedness of the outbreak strain to two pre-outbreak strains
2) The *stx2* RNA transcript levels in: a) the outbreak *E.coli* O104:H4 and two pre-outbreak *E. coli* O104:H4 strains b) *E.coli* O104:H4 cultures subjected to rifampicin, gentamicin, or both at the MIC and MBC levels and c) *E.coli* O104:H4 samples treated with two sub MIC concentrations of rifampicin and gentamicin.
3) The levels of Stx2 in supernatants of: a) *E.coli* O104:H4 exposed to rifampicin, gentamicin, or both at the MIC and MBC levels, and b) *E.coli* O104:H4 cultures grown in two sub MIC levels of rifampicin and gentamicin.
4) The expression of the SOS response activator, the *recA* gene, and repressor, the LexA protein, in *E.coli* O104:H4 samples exposed to two sub MIC concentrations of rifampicin and gentamicin.
5) The efficacy of using rifampicin, gentamicin, or both at the MIC and MBC concentrations to treat BALB/c mice infected with *E.coli* O104:H4.

CHAPTER II

LITERATURE REVIEW

A. General Characteristics of *Escherichia coli*

The *Enterobacteriaceae*, commonly referred to as the "enterics", is a family of gram negative bacteria that includes a wide range of microorganisms that primarily cause gastrointestinal infections. It encompasses more than 40 genera, the most commonly known are *Salmonella, Escherichia,* and *Klebseilla* (1). The genera *Salmonella* and *Escherichia* deviated from a common ancestor about 100-140 million years ago (2). This was followed by the divergence of the *Escherichia* genus into its 5 main species, *Escherichia coli* being the most medically important (3). *E.coli* was discovered in 1885 by the German pediatrician Theodore von Escherich and was known at that time as *Bacterium coli commune,* because it was found in the colon of healthy individuals (4). Nonetheless it was not until 1935, after the incidence of an outbreak of diarrhea in infants that *E.coli* was identified as a causative agent of disease in man (5).

E.coli are rod shaped, non-spore forming, motile, and facultative anaerobe bacteria that can be found in water, soil, and the normal flora of humans and animals (1). They usually grow best at 37 ºC; however, some laboratory isolates have been shown to multiply at temperatures as high as 49 ºC (6). They possess the enzyme oxidase but lack the enzyme catalase (1). Members of this species are mostly lactose, sorbitol, mannitol, xylose, and L-arabinose fermenters. In addition, they nitrate, fumarate, dimethyl sulfoxide, and trimethylamine N-oxide reducers (7).

E.coli is the cause of a wide range of diseases including enteric diseases such as gastroenteritis, community acquired infections such as urinary tract infections, nosocomial infections, and neonatal meningitis (8). In addition, septicemia, pneumonia, and peritonitis are complications of *E.coli* infections (5). *E.coli* strains that produce gastrointestinal diseases can be classified into 5 virotypes. These virotypes are:
Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC),
Enterohemorrhagic *E.coli* (EHEC), Enteropathogenic *E.coli* (EPEC), and
Enteroaggregative *E.coli* (EAEC).

B. Virotypes of *Escherichia coli*

1. *Enteropathogenic E.coli (EPEC)*

EPEC, the first *E.coli* virotype described to cause disease in humans, is usually associated with copious watery diarrhea (5, 9). Additionally, it is the most important cause of diarrhea in infants in developing countries (1, 5). It induces disease by adhering to the small intestine's epithelial cells using EPEC adherence factor (EAF), pili, and intimin. Subsequently, the microvillus is destroyed and the "attachment/effacement" (A/E) histopathology is observed (1). The Locus of enterocyte effacement pathogenicity island ((LEE-PAI) contains the genes that are responsible for the observed histopathology (1, 9). The destruction of the microvilli leads to malabsorption and invasion of host cells and is therefore, partially responsible for the watery nature of the diarrhea (1, 5).

2. *Enteroaggregative E.coli (EAEC)*

The first case of EAEC to be recognized was identified in a child in Peru in 1987 (10). From the time then, EAEC has been associated with persistent watery diarrhea lasting more than two weeks in children and adults, sporadic diarrhea in immune competent adults and infants, and subclinical and chronic infection in the gut leading to mental and physical retardation in children (1, 10). This virotype probably causes disease by attaching to the host cells' epithelial cells in an aggregative manner producing the "stack brick" pattern, producing cytotoxins and enterotoxins, and inducing inflammatory reactions in the body (10, 11).

3. *Enteroinvasive E.coli (EIEC)*

In 1971, DuPont et al. carried out a study showing that certain strains of *E.coli*, termed as Enteroinvasive *E.coli*, were capable of producing an invasive diarrheal disease in volunteers (12). EIEC strains are pathogenically, biochemically, and genetically similar to *Shigella* species. EIEC usually causes disease by invading and multiplying in colonic epithelial cells leading to their destruction (5). EIEC and *Shigella* species share several characteristics including the possession of a 140 MDa plasmid that encodes outer membrane proteins involved in invasiveness and the ability to cause disease characterized by abdominal pain and bloody mucoid diarrhea. EIEC outbreaks are food or water borne, occurring mostly in areas of the world with poor sanitary conditions (11, 13).

4. *Enterohemorrhagic E.coli (EHEC)*

In 1983, a study done by Riley et al. investigating a diarrheal outbreak and another study conducted by Kamali et al. linking Hemolytic uremic syndrome (HUS) with cytotoxin

producing *E.coli* in stools, led to the recognition of EHEC as a distinct group of pathogenic *E.coli* (11). EHEC causes a wide range of illness including mild diarrhea, hemorrhagic colitis, and HUS, a potentially fatal condition. EHEC is characterized by the production of Shiga toxins (Stxs) (9). Like EPEC, EHEC possesses the LEE- PAI that encodes the production of A/E lesions on epithelial cells. In addition, *E.coli* O157:H7, the most common EHEC associated with illness worldwide, harbors a 60 MDa plasmid which contains genes involved in virulence (14).

5. *Enterotoxigenic E.coli (ETEC)*

During the 1950s and 1960s, several studies done by De, Gorbach, and Sack characterized a class of *E.coli* associated with diarrhea, the ETEC (15, 16, 17). ETEC is a major cause of diarrhea in infants in developing countries or in regions where sanitary conditions are deficient. In addition, it is the most important cause of traveler's disease in people traveling to these areas (5). ETEC possesses a number of virulence factors encoded on plasmids including two classes of enterotoxins namely heat liable toxins (LT-I and LT-II) and heat stable toxins (STa and STb), and colonization factors; both are required for disease development (1, 18).

C. Epidemiology of the outbreak of Shiga toxin producing *E.coli* O104:H4

1. *Background of the outbreak*

In early May 2011, the second largest food-borne *E.coli* outbreak started in Germany and subsequently extended to 13 European countries, Canada, and the United States of America (USA) (19, 20). In July 2011, the outbreak was contained and declared "ended" (21).

Globally, 4137 cases including 50 deaths were reported, the majority (96.5%) of which was centered in Germany (22). Other countries that were affected, the number in brackets representing the cases reported in each country, include: Sweden (53), Denmark (26), France (15), Netherlands (11), Great Britain (7), the United States of America (6), Switzerland (5), Austria (5), Poland (3), Luxembourg (2), Spain (2), Canada (1), Greece (1), Norway (1), Czech Republic (1) (20). Shiga toxin producing *E.coli* O104:H4, a novel pathotype Enteroaggregative Hemorrhagic *Escherichia coli* (EAHEC), was identified as the causative agent of the outbreak (19).

2. *Mode of transmission*

Shortly after the onset of the outbreak, epidemiological studies and microbiological examinations were carried out to identify the source and vehicle of the infection. Initially, explanatory detailed interviews, along with geographic and demographic distribution of the cases revealed that the outbreak was food-borne with fecal material of human and/or animal origin being the source of contamination (20, 23). Consequently, studies done by Robert Koch Institute (RKI) in Germany identified fresh raw vegetables as the primary contaminated food source that led to the outbreak (23).

At the beginning of the outbreak, 60 employees in two Frankfurt based companies developed the symptoms associated with the outbreak. As a result, additional extensive investigations and nationwide trace back analysis showed that the canteens of the two companies were linked to an organic farm that produces sprouts in Lower Saxony near Hamburg, through one distributor (20). In addition, it was revealed that these sprouts were distributed to a wide number of the outbreak implicated restaurants, food facilities, and

hotels (24). Furthermore, Shiga toxin producing *E.coli* O104:H4 was detected in feces of infected workers as well as asymptomatic carriers in the Lower Saxony farm (23). Further inspections indicated that the consumption of two sprout seed mixtures was associated with the disease, with fenugreek sprouts found exclusively in both mixtures (25). The fenugreek seeds were traced back to a 15,000 kilogram shipment from Egypt received in December 2009, the majority (10,500 kg) of which was sold to a distributor, who in turn supplied the incriminated German sprout farm. 400 kilogram of the shipment was sold to an English seed distributer that supplied French garden stores (26).

On June 24, 2011, France reported a much smaller outbreak, in which 15 cases with bloody diarrhea were reported. Eleven of the fifteen cases attended an event near Bordeaux two weeks earlier (27). Fenugreek sprouts, served during the event, were traced back to the contaminated fenugreek seeds that were sold to the English distributor (26). It was therefore believed that fenugreek seeds imported from Egypt were the vehicle of infection for both the German and French outbreaks; contamination possibly occurring as a result of using feces as fertilizers or contaminated water for irrigation at the initial production level before importation (23). However, Egyptian officials have denied being the source of this agent. In addition, they did not report the occurrence of any cases of infection with this strain of *E.coli*.

Secondary modes of transmission were the cause of few additional cases that continued to occur even after control and public health measures were taken to prevent further consumption of the contaminated sprouts (23). Person-to-person transmission via close contact especially within families was implicated in the occurrence of new cases. During the outbreak in France, Aldabe et al. reported a case in which an infected man

transmitted the novel *E.coli* O104:H4 to his family (28). In addition, a study done by Hauri et al. identified 6 secondary cases in Germany related to person-to-person transmission within a household (29). Secondary transmissions occur when surfaces, clothings, hands or other body surfaces, and bathroom seats become contaminated with fecal material or vomit from an infected person (20). In addition, asymptomatic carriers of the disease, which were considerable during the outbreak, may have resulted in outbreaks in day-care centers, schools, or nursing homes (23). Likewise, infected or asymptomatic food handlers within households or food industries were also considered to be a possible cause of secondary food contamination during the outbreak (20). One such incident occurred in a school in Germany in which 4 individuals were infected with the outbreak strain. It was subsequently discovered that 3 employees in the catering company that supply the school were asymptomatic carriers (30).

Secondary cases, including a hospitalized man who developed gastrointestinal symptoms after possible contact with feces from an asymptomatic patient within the same ward and a woman who contracted the infection while working in a microbiology lab, were due to nosocomial and laboratory transmission respectively (31). Finally, cases reported in Canada, USA, and European countries with the exception of France, were due to recent travel to affected areas in Germany or contact with these travelers (21).

3. *Incubation Period*

The incubation period for an infection with the outbreak *E.coli* O104:H4 strain ranges between 7-9 days (20). This is considered to be much longer than the incubation period of *E.coli* O157:H7 which usually lasts 3-4 days (32). Its long incubation period possibly

indicates that it has a low infectious dose (30). It is believed that the infectious dose of the outbreak strain could be as low as 100 colony forming units (CFU) (33).

4. *Animal Reservoirs*

While ruminants such as cattle are the major reservoir of EHEC, no animal reservoir has been identified for EAEC (34). The natural host of EAEC strains is considered to be humans, as they have been isolated from most of the world's population. *E.coli* strains exhibiting the aggregative property of EAEC have been identified in feces of infected horses, pigs, and calves in South America; however, these strains were unrelated to the human EAEC strains as they lack their major virulence genes and phenotypic characteristics (35). Whether the outbreak *E.coli* O104:H4 strain, a hybrid pathotype, has an animal reservoir remains undetermined. It has been suggested that humans are the only reservoir for the outbreak strain, since, like EAEC, it seems to be highly adapted to humans (36). Studies conducted shortly after the outbreak concluded that cattle are not the reservoir for the outbreak strain in Germany and France (34, 37). Furthermore, Shiga toxin producing *E.coli* O104:H4 have never been described in animals prior to the outbreak, and have been mainly isolated in humans (20). Additionally, one study illustrated that *E.coli* of the serogroup O104 could be found in feces of cattle in Kansas; yet none of these strains harbored the characteristics of the outbreak strain (38). Another study reported that *E.coli* of serotype O104:H4 was not detected in 593 serotyped Shiga toxin producing *E.coli* (STEC) isolated in food from animal origin (39). Even though cattle are not the reservoir for the outbreak strain, it cannot be concluded that the outbreak strain does not have a possible animal reservoir (36).

5. *Previous occurrence of Shiga toxin producing E.coli O104: H4*

Prior to the outbreak, 8 cases of Shiga toxin producing *E.coli* O104:H4 have been reported globally. These include: 2 cases in Germany in 2001, 1 case in France in 2004, 1 case in Korea in 2005, 1 case in Italy in 2009, 2 cases in the Republic of Georgia in 2009, and 1 case in Finland in 2010 (40, 41). The Finnish and Italian cases were due to travel, the countries of origin being Egypt and Tunisia respectively (40). The sources of the infection for the Korean, French, and German cases were unknown (20). In addition, no food source was identified as the cause of the infection for the Georgian cases (42). None of the Shiga toxin producing *E.coli* O104:H4 isolated prior to the outbreak was identical to the novel strain and differences with it have been reported (43).

D. Clinical manifestation of Shiga toxin producing *E.coli* O104:H4

Infection with the outbreak strain led to a wide variety of clinical manifestations including mild non-bloody diarrhea, hemorrhagic colitis, and HUS. After an incubation period of 7-9 days, infection with Shiga toxin producing *E.coli* O104:H4 during the outbreak resulted in abdominal cramps in 78% of the cases. Like STEC, this was closely followed by non-bloody diarrhea which then became bloody in some cases after 2-3 days. In addition, the gastrointestinal symptoms resolved within 5-10 days with no complications in a high number of cases (44, 45). However, some patients developed HUS 5 days after the onset of diarrhea; a longer period (7 days) is observed in *E.coli* O157:H7 infections (20). Out of the total 4137 cases that were identified during the outbreak, 3241 patients displayed EHEC gastroenteritis and 896 cases developed HUS (22). In addition, the outbreak resulted in 50

deaths, therefore a mortality rate of 1.23% was observed, 2.5 times higher than that of *E.coli* O157:H7 outbreaks (0.5%) (46). 34 of the fatalities were reported in the HUS cases (mortality rate of 3.3%) and the other 16 were reported in the EHEC gastroenteritis cases (mortality rate of 0.5%) (32). Bloody diarrhea was observed in 56% of the EHEC gastroenteritis cases, whereas it was reported in 79% of the patients with HUS (20). About 56% of the patients with EHEC gastroenteritis and more than 90% of the cases with HUS were hospitalized (47). Vomiting (17%) and low grade fever (7%) were the least commonly reported symptoms of the infection with the outbreak strain (32).

Hemorrhagic colitis is an inflammation of the colon characterized by severe abdominal cramp, low grade fever, and bloody watery diarrhea. This condition may progress to HUS. During the outbreak, radiological imaging findings in patients with hemorrhagic colitis differed from that of other hemorrhagic colitis. *E.coli* O104:H4 colitis cases were associated with segmental colitis; this was characterized by the thickening of the descending colon wall and distention of the transverse and/or ascending colon (no thickening). This is different from other *E.coli* colitis in which the transverse colon is mostly affected and the distribution is contiguous (48).

HUS is a condition characterized by a triad of microangiopathic hemolytic anemia, thrombocytopenia, and impaired renal function or failure (49). It is believed that STEC releases the toxins in the intestine, after which they enter the blood circulation. The Stxs then injure the endothelial cells in the gut, kidney, brain, and other organs leading to the generation of thrombin and deposition of fibrin on the microvasculature. As a result Plasminogen activator inhibitor concentration rises, inhibiting fibrinolysis and leading to further accumulation of fibrin in the vessels (50). This leads to microvascular clot,

endothelial edema and leakage, and apoptosis of the endothelial cells. These events are the core pathogenesis of HUS (33).

The risk factors for development of HUS during the outbreak were identified as: elevated leukocyte counts (due to inflammatory response to Stxs), vomiting, old age (due to the possibility of pre-existing renal impairment), and manifestation of visible blood in stools (due to intestinal wall damage enabling bacterial invasion and toxins uptake). Elevated leukocyte counts and vomiting were also identified as risk factors for the development of HUS in other *E.coli* infections (51). In addition, the use of antimicrobial agents in the treatment of *E.coli* O104:H4 infections during the outbreak did not seem to enhance the development of HUS (52, 53).

There were several unusual features observed during the outbreak. First of all, 25% of the reported cases developed HUS. This is an unusually high proportion when compared with outbreaks of STEC, in which HUS usually develops in 8-15% of the cases (54, 55). In addition, whereas HUS occurs primarily in children (the most common cause of renal failure in children**)** and elderly, the majority (88%) of the cases during the outbreak were healthy middle aged adults (median age 43 years) (35, 51). Prior to the outbreak, 1.5%-10% of adults infected with STEC developed HUS in Germany (56). Moreover, the proportion of HUS cases reported in children under the age of 5 during the outbreak was much lower (2%) when compared with previous German STEC outbreaks (69%) (20). Although the reason for these differences is unknown, it might be due to the fact that *E.coli* O104:H4 can better colonize the intestine leading to increased crossing of Stx2 (35). Another distinctive feature of the outbreak is the prevalent involvement of women: 68% of the HUS cases and 58% of the EHEC gastroenteritis cases were women. This might be due to gender

differences in dietary consumption (35). Finally, a high frequency of severe neurological involvements (30%) such as epileptic seizure and encephalopathy were observed in the HUS cases during the outbreak especially when the patients started recovering (45, 57, 58). Neurological symptoms are more common in thrombotic thrombocytopenic purpura (TTP), an auto-immune disorder caused by inhibition of Von Willebrand factor (VWF) protease, ADAMTS13, observed in *E.coli* O157:H7 infections, than in HUS (58).

E. Characteristics of Shiga toxin producing *E.coli* O104:H4

The outbreak strain is a hybrid pathotype that possesses the genomic backbone of EAEC. In addition, the strain acquired virulence factors characteristic of EHEC; thus it was referred to as Enteroaggregative Hemorrhagic *Escherichia.coli* (EAHEC) (59). The typical characteristics of EAEC that the outbreak strain harbored include the ability to produce the "stacked brick pattern" by adhering to Hep2 cells in culture (60), the possession of the EAEC plasmid of aggregative adherence (pAA) which encodes a number of virulence genes, and the capability of producing several Serine Proteases Autotransporter of Enterobacteriaceae (SPATES) involved in intestinal colonization (43) (Discussed in details in section G). Moreover, the outbreak strain acquired several features found in EHEC by gaining mobile elements such as prophages and genomic islands. These virulence factors include: a prophage encoding the *stx2* subtype a gene, an iron uptake system expressed on a high gene pathogenicity island that was initially detected in Yersinia (the *irp2 and fyuA* genes), adhesins such as the structural subunit of long polar fimbriae (LPF) of STEC O26 and O113 (the lpf_{O26} and lpf_{O113} genes), the tellurite resistance loci (the *ter* cluster), and the IrgA homologue adhesion (*iha* gene) which is an outer membrane protein found in

uropathogenic *E.coli* that plays a role in adherence and colonization, biofilm formation, and aggregation (57, 61). The outbreak strain lacked other virulence factors found in EHEC mainly Stx1 (the *stx1* gene), intimin (the *eae* gene), and hemolysin A (the *hly* gene) (27).

The outbreak strain acquired a plasmid carrying the CTM-X-15 and TEM-1 extended spectrum beta lactamase (ESBL) genes ($bla_{CTM\text{-}X\text{-}15}$ and $bla_{TEM\text{-}1}$ respectively) (62). In addition, another plasmid was acquired, which harbors the genes that might be involved in mercury resistance (19).

F. Genome of Shiga toxin producing *E.coli* O104:H4

The genome of the outbreak strain was estimated to have a size of 5.31 Mbp and it comprised about 5215-5225 protein encoding genes. The GC content of the genome was identified to be 50.6% (19). After analyzing the genomes of a number of isolates from the outbreak, it was found out that they differ by only 236 single nucleotide variants (21). In addition, the Pulsed field gel electrophoresis (PFGE) profile of both the German and French isolates was identical. This provides evidence that the outbreak was clonal and single sourced (60). Multilocus sequence typing (MLST) analysis, a procedure that describes the DNA sequence of internal fragments of housekeeping genes (in this case *adk, fumC, gyrB,icd, mdh, purA, and recA*), was carried out on the outbreak strain. Results showed that outbreak strain can be ascribed to the Sequence Type (ST678): *adk*6, *fumC*6, *gyrB* 5, *icd* 136, *mdh* 9, *purA* 7, *and recA* 7 (23) which belongs to the phylogenetic group B1, a very diverse group comprising non-O157, ETEC, EHEC, and commensal *E.coli* (63).

The genome of the outbreak strain was very similar to an EAEC strain that was isolated from an HIV patient with persistent watery diarrhea in Central Africa in 2002,

commonly known as reference strain EAEC 55989 (56, 64). The outbreak strain showed a 99.8% nucleotide identity with the EAEC 55989 strain (65). A study done by Grad et al. predicted that the common ancestor of the outbreak and reference strain existed 30 years ago and the most recent ancestor of the outbreak strain occurred in 2008-2009 (66).

G. Virulence Factors of Shiga toxin producing *E.coli* O104:H4

The high virulence and lethality of the Shiga toxin producing *E.coli* O104:H4 is due to its ability to produce Stx2, the possession of enteroggreggative properties that allow it to adhere to and colonize the intestinal epithelium, and the acquisition of multidrug resistance plasmids and genes.

1. *Shiga toxin 2 (Stx2)*

a. General Characteristics

Shiga toxins produced by EHEC belong to the same family of toxin produced by *Shigella dysenteriae* type 1 (67). In 1977, a group of scientists noticed that some isolates of *E.coli* were able to produce a cytotoxic effect on Vero cell line; however, it was not until 1983 that they were linked to diseases such as Hemorrhagic colitis and HUS (68, 69, 70). There are two Stxs produced by *E.coli* namely Stx1 and Stx2 (71). The Stx1 in *E.coli* and the Shiga toxin in *Shigella* are almost identical differing only in a single amino acid; however, Stx1 and Stx2 share only 56% homology at their amino acids level and 58% homology at their nucleotide level (71).

Stx2 has several variants ranging from Stx2a to Stx2g; these subtypes differ in nucleotide and amino acid sequences (72). Stxs are lethal in mice and rabbits and are

considered enterotoxic in rabbit intestinal segment models (73). Stxs are cytotoxic to several cell lines including Vero, HeLa, Daudi, human B lymphoma, KB, human liver, and human skin fibroblasts (74). In addition, Stx2 was shown to be 100 times more lethal than Stx1 in mice and rabbits (75, 76, 77). Furthermore, strains that produce either Stx1 or Stx1 and Stx2 are less virulent that those that produce Stx2 only (78, 79). A study done in Baboons showed that Stx2, but not Stx1, alone can produce the symptoms of HUS (80)

b. Structure

The Stx (MW 70 kD) belongs to the typical AB5 toxins that are produced by *Vibrio cholera*, *Corynebacterium diphtheriae*, *Bordettela pertussis*, and other microorganisms. The toxin is composed of a single A subunit (MW about 32 kDa), which possesses enzymatic activity, and five B subunits (MW about 7.7 kDa each) which bind to cellular receptors (74). The A subunit can be cleaved by a protease into two portions linked together by a disulphide bond (81). These two domains include A_1 (MW 27.5 kDa), which possesses enzymatic activity, and A_2 (MW 4.5 kDa), the carboxyl terminal that links the A_1 subunit to the B subunit in a non-covalent manner (82).

c. Cellular receptors

The B subunit attaches to glycolipids that have the disaccharide Gal-α(1 -- 4)-Gal terminal moiety belonging to the Globotriosylceramide (Gb3) and Globotetraosylceramide (Gb4) family (82). Due to differences in 9 amino acids in the B subunit and the absence of two amino acids in the C terminal of the A subunit, Stx2e binds to Gb4 more favorably than Gb3, the preferred receptor for all Stx2 variants (83). Gb3 can be found at low levels on

enterocytes and at high levels on endothelial cells including brain, renal, aortic, and glomerular endothelial cells (35). Other sites that might express the Gb3 receptors are: mesangial, lung, and renal tubular epithelial cells, monocytes, astrocytoma cells, platelets, and polymorphonuclear cells (84). In addition, small vessels in the target organs tend to express more Gb3 than other vessels (85). Studies have shown that pro-inflammatory cytokines such as TNF-α and IL-β can increase the manifestation of the Gb3 receptor, thus enhancing the binding of toxins (86).

d. Entry

After the toxin binds to the Gb3 receptor on the cell membrane, a short incubation at 37°C leads to aggregation of toxin-receptor complexes in clathrin-coated pits. The A fragment is then endocytosed and transported through endosomes to the Trans -Golgi network (TGN). In the TGN, the toxin is cleaved by the enzyme furin into the A_1 and A_2 subunits. From the TGN, the toxin is transported through the Golgi stack to the endoplasmic reticulum (ER) where translocation into the cytosol takes place. This is referred to as retrograde transport. If the toxin was not cleaved by furin, then the cytosolic enzyme caplain, may cleave the molecule but this process is usually slower (87, 88). In addition, the two toxins differ in their distribution within the cell: Stx1 is located in the periplasmic space, while Stx2 is found in the extracellular fraction (89).

e. Mode of action

The A_1 subunit is an N-glycosidase that acts by removing an adenine residue in position 4324 in the 5' end of the 28S rRNA component of the 60S ribosomal subunit (90). This

adenine resides on the loop of the rRNA that is essential for the binding of Elongation Factor 1 (EF-1) (82). Therefore, EF-1-dependent aminoacyl tRNA binding to the 60S ribosomal subunit is blocked, which in turn prevents peptide elongation and disrupts protein synthesis leading to cell death (90).

f. Prophage of Shiga Toxin producing *E.coli* O104:H4

Stxs are encoded on lysogenic phages known as lambda phages that can be incorporated into the genome of the microorganism (45). These mobile elements are present naturally in the environment (fecally polluted water, vegetables, and minced meat) (60) and can be gained by horizontal gene transfer among the bacterial population (91). The *E.coli* O104:H4 isolated during the outbreak acquired two lambda-like phages, which share about 67% of their sequence; however, only one of them harbored the *stx* subunits genes (54, 60). The *stx* gene that was obtained was of the variant 2a (43). It has been shown that Stx2a is frequently associated with HUS and hemorrhagic colitis (92). In addition, a study done by Fuller et al. demonstrated that Stx2a is 16-90 more toxic to human kidney cells than HeLa cells (75). The Stx2a in the outbreak strain is 100% similar to the Stx2a found in the *E.coli* O157:H7 strain EDL33 (isolated in Michigan from ground beef in 1983) at the amino acid level, but differs in only a single nucleotide at position 867 (C instead of T) (43, 66). The prophage of the outbreak strain is inserted in the *wbrA* locus of the chromosome (encodes multimeric flavodoxin-like protein) which is also the integration site of the *stx2* gene in the EDL33 strain (65, 93). The *stx2a* gene is integrated between the Q antitermination and S/R lysis genes (60). A study done by Brzuszkiewicz (19) showed that the prophage of the

outbreak strain is most closely related to the VT2phi_272 bacteriophage found in *E.coli* O157:H7 strain 71074, a hyper-virulent strain linked to severe human diseases.

The prophage is usually in a lysogenic and repressed form in the genome and the transcription of the toxin genes is silenced by the cI repressor (represses P_L and P_R promoters) (94). The toxin genes are late genes of the prophage and are activated only during the lytic phase (95). Agents that destroy the DNA result in a decrease in the cI protein leading to the initiation of the lytic cycle, the induction of the phage, and the production of the toxin (94). Initially, the cI repressor is cleaved at the connector region that holds the C terminal to the N terminal region resulting in a regulatory cascade of gene activation (89). The P_L and P_R promoters then initiate the expression of intermediate early genes that lead to the production of two proteins namely the N antitermination factor and cro (inhibits cI synthesis). The N antitermination protein expresses delayed early genes that encodes the Q antitermination protein and results in the removal and replication of the prophage. The Q antitermination protein binds to the DNA site known as *qut*, which then allows the transcription of late genes (toxin genes and lysis genes) (96).

There is a wide range of *stx* prophage inducers including PH, iron deficiency, hydrogen peroxide, and antimicrobial agents such as quinolone and trimethoprim (97, 98, 99). In addition, Mitomycin C, a chemotherapeutic agent that damages the DNA by forming crosslinks, induces the *stx* prophage by activating a repair mechanism known as the SOS response. Moreover, agents such as ultraviolet radiation (UV) that induce mutations in the DNA can also activate the SOS response and induce the *stx* prophage (100).

2. *Enteroaggreggative Characteristics*

Although many virulence factors have been identified in EAEC, no particular factor has been constantly isolated in all pathogenic strains (101). A primary virulence factor that is responsible for the "stacked brick" phenotype, colonization and adherence to the intestinal mucosa, and hemagglutination of human erythrocytes (102), is an adhesin known as Aggregative Adherence Fimbriae (AAF) whose genes are located on the virulence plasmid, pAA (103). There are four variants of AAF that differ genetically and morphologically; they have been isolated in different prototypical strains (14) and include: AAF/I, AAF/II, AAF/III, and HUS-associated Diffuse Adherence (Hda) (35). The outbreak strain possessed a pAA plasmid encoding the AAF/I that have been originally described in the EAEC strain 17-2. The AAF/I belong to the Dr Family of adhesins, a group of adhesins that bind to the blood antigen "Dr", that are usually linked to diarrheagenic and uropathogenic *E.coli* (104). AAF/I are elongated, thin, and elastic adjunctions that have a diameter of 2-3 nm and form loose bundles of filaments (105). The genes that encode the AAF/I subunits are arranged in two regions separated by a 9 kb DNA. The first region contains four genes in the *agg* operon arranged in the following order from left to right: the *aggD* gene (encodes the periplasmic chaperone protein), the *aagC* gene (encodes the outer usher protein), the *aggB* gene (encodes the invasion protein), and the *aggA* gene (encodes the structural subunit of the fimbria), all of which are involved in the production and assembly of the filaments (106). Region 2 harbors the gene of the plasmid regulator, *aggR*, which controls the expression of the fimbria (107).

Another virulence factor located on the pAA plasmid acquired by the outbreak strain is dispersin. This protein is encoded by the *aap* gene (antiaggregation protein)

located upstream of the *aggR* gene (controls it) (108). Dispersin is a low molecular weight surface coat protein (MW 10.2 kDa) that interacts with the lipopolysaccride (LPS) and controls the AAF/I's structure. It works by neutralizing the LPS's negative charge and allowing the positively charged AAF to be deposited (60, 109, 110). This protein is essential for the proper function of AAF and its absence leads to impaired adherence to the intestine (60). In addition, dispersin scatters the EAEC on the intestinal mucosa which in turns spreads the infection (101). Dispersin is released out of the bacterial cell using the ABC transport system (*aat* encoded secretion system) encoded by the *aatPABCD* cluster genes (14). Another cluster of genes that might play a role in the adhesion of the bacteria includes the *aggR* activatied island genes (*aai*). This cluster of genes (16 genes) is located on a pathogenicity island known as pheU which is found in the chromosome. It contains open reading frames that are regulated by the *agg*R gene (111). These genes form the type 6 secretion system that delivers toxins into host cells or other bacteria (112).

The *aggR* gene is a transcriptional activator that belongs to the AraC family of gene regulators which are needed to express fimbria in some *E.coli* strains (107). Strains containing the *aggR* gene are known as typical EAEC, since its presence is linked to diarrheal disease (14, 113). In addition, EAEC strains of animal origin are classified as atypical, since they lack the *aggR* gene (114). The *aggR* gene, the master regulator of virulence genes, is also located on the pAA plasmid and has been shown to control the expression of 44 genes including 16 hypothetical genes, chromosomal genes such as the type 6 secretion system genes, and plasmid genes such as the fimbrial biosynthesis genes and disperin and its secretion system genes (113).

The outbreak *E.coli* O104:H4 strain can produce cytotoxins or enterotoxins such as plasmid encoded toxin (Pet) and Shigella enterotoxin 1 (Set 1) (103). However, the outbreak strain does not possess the plasmid located gene, *astA*, that encodes a small heat stable enterotoxin, *Escherichia coli* heat-stable enterotoxin 1 (EAST-1), found in some strains of EAEC, including the 55989 reference strain. Pet is a serine autrotranspoter protein that has a molecular weight of 108 KDa and acts as both a heat liable enterotoxin and a cytotoxin (115). Pet is encoded by a gene located next to the AAF biosynthesis gene on the pAA plasmid (59). Unlike all the pAA encoded virulence factors, Pet is not controlled by the *aggR* gene (103). Pet acts by cleaving spectrin, a constituent of the membrane cytoskeleton, leading to cytoskeletal rearrangement. In addition, Pet was recognized as an enterotoxin and a cytotoxin in rat jejunal tissue in Ussing chambers (116). It results in elongation, rounding, detachment, and exfoliation of cells (117). Pet can also cause mucosal destruction, enhanced mucus secretion, and production of crypt abscesses (116). Set 1, on the other hand, is an AB5 type toxin encoded by the *set 1* gene (118). It is composed of a 20 kDa enzymatic A subunit (*set A* gene) and 5 7kDa B subunits (*set B* gene) (119). Set 1 was initially isolated in *Shigella flexini 2a* and it is toxin is identical to the Set 1 toxin identified in EAEC. Set 1 was shown to induce fluid accumulation in rabbit ileal loops (118).

The outbreak stain's genome encodes SPATES; these are extracellular proteins that play a role in the colonization and damage of the intestine (21). They include protein involved in colonization (Pic), *Shigella* extracellular protein A (SepA), *Shigella* IgA protease-like homologue (SigA), and Pet (21). The Pic protein is associated with mucus hypersecretion and mucoid diarrhea. This protease is present in *Shigella flexini* and EAEC

strains (60). The *set 1* and *pic* genes are located in the bacterial chromosome on a pathogenicity island called SHI; together they play a role in disease establishment (59, 60, 119). The Pic protein (MW 109 kDa) has several functions which include the breakdown of the intestinal epithelial cell protein, mucin (aid in colonization of the mucosa), resistance of serum, promotion of hemagglutination, and inactivation of the compliment cascade (110, 120). SigA, a 140 kDa protein, is another SPATE found in EAEC and *Shigella* strains that causes erythrocyte agglutination, triggers the "rounding up" of Hep-2 cells, and degrades fodrin, a protein that links the microfilaments and plasma membrane in non-muscle cells (121, 122, 123). SepA, a 110 kDa plasmid encoded protein, stimulates inflammation and aid in intestinal invasion (124). EAEC strains usually contain two SPATES, however, the outbreak strain can produce 3 SPATES (Pic, SepA, and SigA), an unusual phenomenon. Therefore, the presence of three SPATES and the enhanced adherence of the bacteria to the intestine that facilitate the increased absorption of Stx2 might explain the high virulence of the outbreak strain (21).

3. *Resistance profile*

All isolates from the outbreak exhibited the ESBL phenotype due to the acquisition of an 89 kb plasmid harboring the bla_{TEM-1} and $bla_{CTX-M-15}$ genes, making them resistant to penicillins, for example Ampicillin, and third generation cephalosporins such as Cefoxitin, Cefotaxim, Ceftazidime (20, 66). Prior to the outbreak, only two STEC strains displayed the ESBL phenotype; both were STEC O26:H11 which expressed the CTX-M-18 and CTX-M-3-type ESBL (32). The outbreak strain was resistant to other antimicrobial agents including Streptomycin, Tetracyclin, Trimethoprim/Sulfamethoxazol, and Nalidixic acid.

Another feature that was acquired by the outbreak strain is tellurite resistance. Tellurite resistance, common in *E.coli* O157:H7, is encoded by the operon *terZABCDEF* located on a pathogenicity island on the chromosome. Furthermore, the outbreak strain might bear a mercuric resistance plasmid, as all genes involved in mercury resistance were identified in the bacteria (19). These includes the regulators (the *MerR* and *MerD* genes), ion transport (the *MerT, MerP, MerC* genes), and mercuric ion reductase *(*the *MerA* gene*)* genes (Bloch, 2012). Finally, a study done by Mellmann et al. showed that *E.coli* O104:H4 harbored the rpoS gene, a regulator of stress response such as acid resistance. In addition, a higher number of *E.coli* O104:H4 cells survived pH 2.5 conditions in comparison to the EDL933 strain (62).

H. SOS response

In 1975, Miroslav Ramdan concluded that *E.coli* harbored a DNA repair system, which was termed the "SOS response" after the international telegraph danger signal in Morse alphabets "SOS". This was due to the observation that the lambda phage was induced, filaments were formed, and UV irradiated lamda phages were re-activated, in *E.coli* treated with UV radiation (125). The SOS response, a regulatory network widespread among bacteria, is a response to DNA damage which leads to a halt in DNA replication, allowing the bacteria to maintain the correct structure of the DNA, endure DNA damage, and restart replication before it dies (125, 126, 127).

The SOS response system is made up of more than 50 genes located at different sites on the chromosome (128) including *lexA* (transcriptional repressor of the SOS response genes), *recA* (inducer of the SOS response), *recQ* (helicase and ATPase that stops

erroneous recombination), *recN* (recombinational repair), *sulA* (inhibits cell division), *umuD* and *umuC* (interact with each other and play a role in SOS mutagenesis; component of Polymerase V (Pol V)), *uvrA*, *uvrB*, and *uvrC* (part of UvrABC endonuclease that plays a role in excision repair), *uvrD* (Helicase II required in excision repair), *ruvA* (interacts with *ruvB* and recognizes Holliday junctions), *ruvB* (branch migration helicase), *ssb* (binds to single stranded DNA (ssDNA)), *dinI* (inhibits cleavage of UmuD and stabilizes RecA filaments), *dinB* (DNA polymerase IV that plays a role in adaptive mutation), and many others (129, 130, 131).

The SOS response usually induces several mechanisms in response to DNA damage in order to promote survival and repair. These include excision repair, recombinational repair, and mutagenesis (132). The excision repair occurs when bulky lesions caused by thymidine dimers or pyrimidine photoproducts are present (133). The uvrA and uvrB proteins form the complex $uvrA_2B$ which delivers the uvrB to the damaged site. Subsequently, the uvrB forms a complex with the DNA, which then attracts the uvrCa protein. This in turn causes conformational change in the urvB leading to the cleavage of a phosphodiester bond in the lesion. UvrD removes the excised segment, polymerase I (Pol I) fills the gap in the DNA, and ligase seals the area (134). The SOS response also functions in recombinational repair which usually occurs when a replication fork is halted prior to replication termination (135). Thus, the SOS response is induced when single stranded gaps appear at the stalked fork. Initially, the RecBCD complex processes one end of the DNA, forming a single strand extension in the 3' end strand. RecA then binds to the ssDNA and aligns it with the double stranded DNA (dsDNA) in search for homology (136). Subsequently, the RuvA and RuvB proteins form a complex which then stabilize the

branched DNA structure especially a Holliday junction (four stranded junction) and promote the migration of the junction (135).

When the DNA polymerase III (Pol III) encounters an unrepairable lesion, DNA replication stops and an error prone repair mechanism, the SOS mutagenesis, ensues (129). Three enzymes, Polymerase II (*polB*), IV (*dinB*), and V (*umuC and umuD*), play a role in a process known as translesion DNA synthesis. In this process, the lesion is bypassed and replication is resumed (137). RecA stimulates the autocleavage of UmuD into UmuD', which in turn interacts with UmuC to form the complex, $Umu(D'_2)C$ (Pol V). This is then targeted to the DNA lesion by the activated RecA. The complex acts as an elongation factor, aiding the halted DNA Pol III in the insertion of nucleotides opposite the DNA lesion and resuming DNA synthesis (127, 129).

The SOS response has a large variety of roles other than DNA repair. A study done by Mellies et al. showed that the SOS response controls the genes in the type III secretion system found in EPEC (138). The SOS response also plays a role in the horizontal transfer of resistance genes through mobile elements. One such example occurs in *Vibrio cholera* which usually harbors SXT, an Integrative Conjunctive Element. This mobile element provides resistance to a number of antibiotics such as streptomycin, sulphamethoxazle, chloramphenicol, and trimethoprim (139). A study done by Beaber et al. showed that upon DNA damage, the *SetR* gene, an SXT encoded repressor, is autocleaved by the SOS response system leading to enhanced expression of the *setC* and *setD* genes which usually control the STX conjugal transfer and integrase genes (139, 140). Finally, the SOS response provides RecA, which induces the autocleavage of the cI repressor in *E.coli*. This

transforms the lambda phage from the lysogenic to the lytic state and enhances the expression of genes harbored by the phage such as the toxin genes (132).

The SOS response can be induced by a wide variety of factors that result in DNA damage or DNA synthesis arrest, and consequently lead to the accumulation of ssDNA (125). These factors include pH changes, heat, high pressure, oxidative stress, nutritional starvation, antimicrobial agents, Mitomoycin C, methyl methanesulphate (MMS), and UV radiation (125, 141).

1. *RecA Coprotease (recombinase A)*

RecA is a multifunctional protein that is composed of 3 mains domains: a large central core called the RecA fold, a domain at the N terminal, and another at the C terminal (130). In addition, it is made up of 352 amino acids conferring a molecular weight of 36 kDa (125, 130). The RecA protein is a ubiquitous, highly conserved protein found in almost all bacteria (131). Moreover, it is synthesized in very large amounts, reaching about 10000 copies per cell at constitutive levels and increasing 10 folds after SOS induction (125). RecA is present in two forms: the active and the inactive state (129). Initially, RecA binds to the ssDNA and forms a helical nucleoprotein which contains 3 bases per monomer/ 6.2 monomers per turn. The difference between the active and inactive form is the helical pitch size which depends on the availability of ATP. When ATP binds to the nucleoprotein, the bound DNA stretches and forms the extended conformation, with a large helical pitch, needed for the proper function and activation of the RecA protein (125).

RecA is mainly involved in DNA recombination repair, cell division, and mutagenesis (129). In addition, the activated RecA protein acts as a co-protease by

stimulating the autocleavage of a number of proteins including the lexA, UmuD, and cI protein (126).

2. LexA repressor (locus for X-ray sensitivity A)

LexA protein is the primary transcriptional repressor of the genes involved in the SOS response (132). The SOS genes or din genes (SOS induced damage-inducible) harbors a palindromic 20 nucleotide long SOS box located near the promoter or operator site (125). In an undamaged cell, the 27 kDa LexA protein binds as a homodimer to the SOS box, limiting the access of RNA polymerase to the promoter site and repressing the expression of the genes (125, 142). All SOS genes have one SOS box except LexA itself and RecN which possess 2 boxes and 3 boxes respectively (142, 143). In addition, LexA is 80 percent repressed under normal conditions (143). The strength of the binding and affinity of LexA to the operator regions determines the degree of repression and the timing of the de-repression (127). The LexA protein is made up of 202 amino acids which make up two binding domains, the N terminal DNA binding domain (NTD) joined by a short hydrophilic region to the C terminal catalytic domain (CTD) which is responsible for LexA dimerization and cleavage (144, 145, 146). After DNA exposure to damaging agents, RecA binds to ssDNA in the presence of ATP and becomes activated. This stimulates the autocleavage of LexA between the Ala^{48}-Gly^{85}bond (cleavage site) present in the CTD. Consequently, the binding of the DNA to the NTD is weakened (145, 146). The LexA protein decreases 10 fold and the SOS genes become induced at different times and levels (131). LexA binds weakly to the RecA promoter region inducing it immediately (less than 1 minute) after exposure to high doses of UV (127). Other rapidly induced genes are the

lexA (a SOS gene itself), *uvrAB*, *ruvAB*, and *urvD* genes (125). If the damage persists, genes whose operator binds tightly to LexA (the *sulA* and *UmuCD* genes) are de-repressed (latest stage of SOS induction) to ensure that DNA repair occurs (125). After the DNA is repaired, the cellular concentration of the LexA protein increases and repression of the SOS response is re-established (129).

I. Identification of Shiga toxin producing *E.coli* O104:H4

1. *Detection of Shiga toxin producing E.coli O104:H4 in stool specimens*

Although there is not a standard method for the detection of non O157 STEC, the most reliable procedure for identification of the outbreak Shiga toxin producing *E.coli* O104:H4 in stool from patients is based on a combination of detection methods. The first class of methods is based on biochemical and microbiological characteristics of the microorganism. After enriching the fecal specimen in an appropriate broth (such as Gram Negative (GN) broth), the clinical specimen is plated on selective media such as the Cefixime Tellurite Sorbitol MacConkey agar (CT-SMAC), or ESBL agar plates such as the Trytone Bile X-Glucuronide medium (TBX); both contain antibiotics that allow the growth of the outbreak strain and inhibit the majority of other strains, making it easier to get a pure culture and isolate the pathogen (43, 57). Unlike *E.coli* O157:H7, the outbreak strain is a sorbitol fermenter and appears reddish-pink on CT-SMAC agar (32). Positive results on culture plates are further tested by immunological assays, a second group of methods that can be used. This aims at determining virulence factors such as the O somatic and H flagella antigens. These immunoassays include enzyme-linked immunosorbent assays (ELISA), immunoblot assays, Reversed Passive Latex Agglutination (RPLA), Enzyme Immunoassay

(EIA), and Western Blot (59). In addition, immunological assays can be used to detect Stx2 using Shiga toxin detection kits (43). Molecular methods, the third group of methods, can be done in parallel with immunological assay methods. These include Polymerase Chain reaction (PCR) and Real-time PCR which target virulence genes such as the *stx2, eae,* and O and H antigens genes (*rfbO104 or wzxO104* and *fliCH4*). A multiplex PCR can also be used to detect multiple genes namely the *stx2*, and/or *eae*, *rfbO104*, and *fliCH4* genes (147). In addition, PCR targeting EAEC and EHEC characteristics such as the AAF/I cluster genes and the *stx2* gene respectively can be used as a rapid and specific tool to identify Shiga toxin producing *E.coli* O104:H4 (148). During the outbreak, the STEC detection techniques were not designed to deal with the outbreak pathogen. Therefore, it is necessary to develop more universal methods that can be applied to new unusual strain (59)

2. *Detection of Shiga toxin producing E.coli O104:H4 in food samples*

A method using RT-qPCR was proposed for the isolation and detection of the outbreak strain in food by The European Union Reference Laboratory for STEC. The food sample had to be 25 g or 50 g to enhance the sensitivity of the detection method (23). For the isolation of the outbreak strain, the food samples such as seeds (implicated in the outbreak) were ground and enriched in Buffered Peptone water (225 ml for 25 g). This was followed by incubation for 18-24 hours at 37 ºC. 1 ml of this suspension was vortexed, centrifuged, and the supernatant was used for DNA extraction and purification. This was followed by Real-time PCR for screening and detection of the *stx2* gene. Positive results demonstrating the presence of the *stx2* gene were tested for the presence of the O somatic antigen (*wzxO104*gene) using Real-time PCR. The positive *wzxO104* Real-time PCR results

(presumptive presence of Shiga toxin producing *E.coli* O104) were streaked on either MacConkey or TBX agar plates and another medium supplemented with antimicrobial agents. The isolated colonies were subsequently tested for the presence of the *stx2* gene. Colonies that showed positive *stx2* Real-time PCR results were further tested for the O104 and H4 antigens genes for confirmation (*wzxO104* and *fliCH4* genes). Alternatively, the isolated pathogen might be subjected to multiplex PCR that screens for the presence of the *stx2, TerD* (tellurite resistance), *rfbO104*, and *fliCH4* genes to confirm the virulence profile of the outbreak strain (149).

During the outbreak, *E.coli* O104:H4 was not isolated from the suspected seeds in the implicated farm. This could be explained by the fact that: the contaminated seeds were no longer in stock and the amount of the bacteria in the food samples were too low and unevenly distributed, making it difficult to detect the microorganism by the diagnostic methods. In addition, the detection and isolation of *E.coli* O104:H4 from the seeds was complicated by the ability of the pathogen to enter a viable but non-culturable state (VBNC). This condition is characterized by the inability to grow on culture media despite the presence of viable cells. The strain enters this state under stressful conditions such as lack of nutrients, tap water, and toxic concentrations of copper ions (23, 59).

J. Treatment of Shiga toxin producing *E.coli* O104:H4

The treatment of infection with Shiga toxin producing *E.coli*, including *E.coli* O104:H4 is mainly supportive. Early protection of renal function, maintenance of renal blood flow, and initiation of volume expansion is usually a key to prevent development of complications such as HUS (32, 33). Fluid balance should be maintained and rehydration using isotonic

solutions should be sustained (33, 50). Antimotility agents which inhibit peristalsis, delay clearance of the organism, and poses a risk factor for progression to HUS, should be avoided (150, 151, 152). In addition, narcotics and non-steroidal drugs, which diminish renal blood flow, should not be used (32). In case of development of HUS, treatment is still controversial. However, management is mainly based on sustaining electrolyte balance, monitoring central and peripheral pressure, and controlling cardiac function (45). Treatments such volume replacement, erythrocyte and platelet transfusion, and hemo-or peritoneal dialysis for kidney injury target renal, gastrointestinal, hematological and vascular complications (153).

Some novel therapies are being designed to prevent development of HUS. These include probiotic bacteria, Shiga toxin binders (such as Gb3 mimic), and active vaccines (using Stxs or protective antigens) (50, 153). In addition, several forms of therapy have been developed in order to prevent complications of STEC infections, some of which have been applied during the outbreak. For instance, monoclonal antibodies have been developed against proteins such as Stx1, Stx2, and others that play a role in the development of HUS. A specific human monoclonal antibody developed against the A subunit of Stx2 showed an 85% survival rate in piglets infected with *E.coli* O157:H7 (155). In addition, HUS can be treated by eculizumab, a human monoclonal antibody that is targeted against the terminal complement protein, C5. This prevents the formation of C5a and subsequently the membrane attack complex which usually intensifies vascular damage upon exposure to Stx (60, 156, 157, 158). Encouraged by the success of treating three infants during the 2011 outbreak with eculizumab, the antibody was given to about 300 patients (157). However, a study done by Menne et al. showed that patients who received eculizumab during the

outbreak did not show significant improvement (156). Another mode of treatment that was used during the outbreak was plasma exchange. This was recommended by the German Society of Nephrology for HUS cases with severe neurological and renal complications (156). It is believed that plasma exchange removes toxins and antibodies against the blood clotting enzyme, ADAMTS13 (45). However, several lines of evidence contradict these assumptions including: Stxs are hardly found in the circulation, the concentration of Stxs in the colon decreases after development of HUS, and most microvascular damage is done before symptoms appear (159, 160). During the outbreak, the benefits from plasma exchange therapy were marginal (154).

The use of antimicrobial agents in the treatment of STEC is controversial due to its association with an increased risk of HUS development. *In vitro* trials have demonstrated that the use of certain antimicrobial agents, mainly the quinolones, trimethoprim, and furazolidone, appears to augment the production of Stxs in STEC strains such as *E.coli* O157:H7, presumably due to bacterial lysis and subsequent release of toxins from the periplasmic space. This enhanced release of toxins may alternatively be due to the induction of the *stx* prophages harbored by the bacterium, activated by the SOS response (97). Numerous studies have been conducted to determine the effect of different antimicrobial agents on Stx release in *E.coli* O157:H7 and consequently the development of HUS. In general, these studies indicate that macrolides, aminoglycosides, carbapenems, rifampin, rifaximin, and fosfomycin either had no effect or suppressed Stx2 production while fluoroquinolones, trimethoprim-sulfamethoxazole, and ampicillin enhanced Stx2 production (161). A study done in the Department of Experimental Pathology, Immunology and Microbiology indicated that using rifampicin, an agent that inhibits DNA-

dependent RNA polymerase and prevents the initiation of RNA synthesis, before using gentamicin, an aminoglycoside that inhibits protein translation by binding to 16S rRNA component of the 30S ribosome subunit, led to a better survival rate in a mouse model infected with *E.coli* O157:H7.

During the 2011 outbreak, the Centers for Disease Control and Prevention (CDC) recommended not to use antimicrobial agents for the treatment of *E.coli* O104:H4. In addition, the German Society of Infection (DGI) recommended the use of: carbapenems in case of invasive complications, rifaximine for eradication of the pathogen from the intestinal tract, and macrolides for elimination of nasopharyngeal meningococcal pathogens before eculizumab therapy (162). Shortly after the outbreak, several studies were conducted to assess the effect of antimicrobial agents on disease progression. A study done by Geerdes-Fenge et al. showed that antimicrobial agents such as meropenem and ciprofloxacin decreased the risk of death and seizures in HUS patients (53). In addition, the incidence of long term carriage of *E.coli* O104:H4 was reduced in patients treated with azithromycin (163). A study done by Menne et al. demonstrated that antimicrobial agents such as ciprofloxacin and meropenem were beneficial in *E.coli* O104:H4 infected patients who developed HUS. However, whether the therapeutic use of antimicrobial agents is a risk factor for development of HUS is still a debatable issue.

CHAPTER III

MATERIALS AND METHODS

A. Source of *Escherichia coli* O104:H4 strains

The outbreak Shiga toxin producing *E.coli* O104:H4 strain D3774/C22711 was obtained from Statens Serum Institut in Denmark. This strain was isolated during the 2011 outbreak. In addition, two pre-outbreak Shiga toxin producing *E.coli* O104:H4 strains, 2009EL-2050 and 2009EL-2071, were acquired from CDC. These strains were isolated from cases with bloody diarrhea in the Republic of Georgia in 2009.The strains were stored in Brucella broth (Becton, Dickinson & Co., Sparks USA) with 10% glycerol (Sigma Chemical Co., St. Louis, MO) at -80 ºC at the Department of Experimental Pathology, Immunology, and Microbiology. For short term storage, the samples were cultured on MacConkey agar plates and kept at 4 ºC for utmost 2 weeks, after which they were re-cultured.

B. Antimicrobial Agents

Rifampicin was obtained in the crystalline form, had a purity of 97%, and was stored at -20 ºC (Sigma Chemical Co, St. Louis, MO). A Concentrated stock solution was prepared based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. The solvent employed was methanol and the diluent used was water. A mass of 104.6 mg was dissolved in 9.9 ml methanol to obtain a concentration of 10240 µg/ml. 500 µl aliquots of the stock solution were prepared and stored at -80 ºC.

Gentamicin was acquired in a crystal like form, had a potency of 599 µg/ml, and was kept at 4 ºC (Sigma Chemical Co, St. Louis, MO). A Concentrated stock solution was

obtained based on CLSI guidelines. The solvent and diluent was water. To obtain a concentration of 5120 µg/ml, a mass of 85.4 mg was dissolved in 10.4 ml water. 500 µl aliquots of the stock solution were prepared and stored at -80 ºC.

C. Minimum Inhibitory Concentration (MIC) Determination

Broth dilution method was performed to determine the MIC of rifampicin and gentamicin for the outbreak *E.coli* O104:H4 strain. A colony of *E.coli* O104:H4 was taken from a MacConkey agar plate, inoculated into a 2 ml Mueller Hinton II (MH II) broth tube, and incubated overnight at 37 ºC. A turbidimeter (Densimat, Biomerieux, France) was used for the measurement of bacterial growth. A 0.5 McFarland reading was taken to be equivalent to 10^8 CFU/ml. Therefore, to get a reading of 10^6 CFU/ml bacterial concentration, a 100 fold dilution of the 0.5 McFarland bacterial suspension was performed.

For each drug, one ml of MH II broth was added to 13 labeled tubes. One ml of the antimicrobial agent, with an initial concentration 512 µg/ml, was added to the first tube. Subsequently, the antimicrobial agent was subjected ro a 2 fold serial dilution. The last tube had no antimicrobial agent added (served as control for bacterial growth). This resulted in a 2 fold dilution in the concentration of the antimicrobial agent in each tube. One ml of the 10^6 CFU/ml bacterial suspension was then added to each tube. As a result, the final volume in each tube was 2 ml and the final concentration of the antimicrobial agent in tubes 1-13 were: 128 µg/ml, 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml, 0.0625 µg/ml, 0 µg/ml respectively. In addition, the final concentration of *E.coli* O104:H4 in each tube was 5×10^5 CFU/ml. The tubes were incubated overnight at 37 ºC. The lowest concentration of the antimicrobial agent that

prevented visible bacterial growth, observed as “no turbidity” in the tube, was considered as the MIC.

D. Minimum Bactericidal Concentration (MBC) Determination

For each antimicrobial agent, all tubes that did not show visible growth (lack of turbidity) in the MIC assay were used for determining the MBC. 0.5 ml was taken from each of these tubes and added to another tube containing 2 ml MH II broth. This resulted in a 5 times dilution, therefore, the concentration of *E.coli* O104:H4 was 1×10^5 CFU/ml of in each tube. Consequently, 0.2 ml of this suspension was spread onto a MacConckey agar plate and incubated overnight at 37 ºC. The final cell count of the bacteria inoculated onto the agar plates was 2×10^4 CFU. The MBC of an antimicrobial agent is the lowest concentration of the drug that results in a 99.9% reduction in the initial bacterial density. Since the inoculum from the non-turbid MIC tubes contained 2×10^4 CFU of the bacteria, the agar plate for each antimicrobial agent that contained 20 colonies or less (0.01% of 2×10^4 CFU) was considered as the MBC.

E. DNA extraction

DNA was extracted from the outbreak and the two pre-outbreak *E.coli* O104:H4 strains using the illustra bacteria genomic Prep Mini Spin kit (GE Healthcare, UK) according to the protocol for purification of genomic DNA from gram negative bacteria.

1. *Materials needed (provided by the kit)*:

- Lysis buffer type 2
- Lysis buffer type 3
- Lysis buffer type 4
- Wash buffer type 6
- Elution buffer type 5
- Proteinase K enzyme (30 mg was dissolved in 1.5 ml nuclease free water, giving a final concentration of 20 mg/ml; this was stored at 4 ºC).

2. *Protocol:*

a. Collection of bacterial culture

- Few colonies from a fresh culture of each of the three *E.coli* O104:H4 strains grown on MacConkey agar plates, were inoculated in 3 separate tubes containing Luria Bertani (LB) broth (Becton, Dickinson & Co., Sparks USA) and incubated overnight at 37 ºC. The next day, 1.5 ml of each bacterial suspension was centrifuged at 16000 × g (full speed) for 30 seconds. The supernatant was aspired without disturbing the pellet at the bottom of the tube.

b. Lysis

- To each bacterial pellet, 40 µl of Lysis buffer type 2 was added. The solution was mixed immediately by vortexing until a homogenous mixture was obtained (no visible pellet).

- 10 µl of Proteinase K (20 mg/ml) was added to each of the samples which were then vortexed for 10 seconds.
- 10 µl of Lysis buffer type 3 was added to each of the samples and the solutions were vortexed for 10 seconds and spun for 5 seconds to collect the samples at the bottom of the tubes.
- Incubation of the samples was carried out at 55ºC for 15 minutes; halfway through (at minute 7) and at the end of the incubation (at minute 15) the samples were vortexed and spun for 5 seconds.

c. Purification

- 500 µl of Lysis buffer type 4 was added to each sample and the samples were vortexed for 10 seconds and incubated for 10 minutes at room temperature. At minute 5 and minute 10, the samples were vortexed and spun for 5 seconds to collect the samples at the bottom of the tubes.
- Each sample was transferred to a mini column placed inside a collection tube and spun for 1 minute at 11,000 × g.
- The flowthrough in each collection tube was discarded and the mini columns were placed back in their respective collection tubes.

d. Wash and Dry

- 500 µl of Lysis buffer type 4 was added to each column which was then spun for 1 minute at 11,000 × g.

- The flowthrough in each collection tube was discarded and the mini columns were placed back in their respective collection tubes.
- 500 µl of Lysis buffer type 6 was added to each column and the samples were centrifuged for 3 minutes at 16,000 × g.
- Each collection tube was discarded and each column was transferred to a DNase free 1.5ml microcentrifuge tube.

e. Elution

- 200 µl of Elution buffer type 5, pre-heated to 70ºC, was added to the center of each column and the samples were incubated at room temperature for 1 minute.
- Each sample was centrifuged for 1 minute at 11,000 × g and the eluted genomic DNA, the flowthrough, was collected.

f. DNA concentration measurement and storage

- Using a spectrophotomer, the concentration of each DNA sample (20 µl of DNA diluted in 480 µl distilled water) was measured at a wavelength of 260 nm.
- The DNA samples were stored as 10 µl aliquots at -20 ºC.

F. Polymerase Chain Reaction

Polymerase Chain Reaction was carried out in order to determine if the *stx2* gene was present in the outbreak and the two pre-outbreak *E.coli* O104:H4 strains. The amplicon size of the *stx2* gene is 83 base pair (bp).

1. ***Materials needed:***

- 10x Taq DNA polymerase buffer with $(NH4)_2SO_4$ (Fermentas, USA)
- 25 µM Magnesium chloride (Fermentas, USA)
- 2 mM dNTPs (a 500 µl mixture was prepared by adding 10 µl of 100mM dATP,10 µl of 100mM dGTP, 10 µl of 100mM dTTP,10 µl of 100mM dCTP and 460 µl nuclease free water) (Fermentas, USA)
- Nuclease free water (Amresco, USA)
- 5U/µl Taq DNA polymerase (Fermentas, USA)
- The extracted DNA (diluted to become 10 µg/ml)
- Forward *stx2* primer (Thermo Scientific Inc., USA)
    - Sequence: GAT GTT TAT GGC GGT TTT ATT TGC
    - The Primer in the lyophilized form was reconstituted with a certain amount of 1x TE buffer (Amresco, USA) according to the manufacturer protocol (Amresco, USA) to obtain a 100µM stock solution.
    - Aliquots of 25 µM were prepared and used in the PCR mixture.
- Reverse *stx2* primer (Thermo Scientific Inc., USA)
    - Sequence: TGG AAA ACT CAA TTT TAC CTT TAG CA
    - The Primer in the lyophilized form was reconstituted with a certain amount of 1x TE buffer (Amresco, USA) according to the manufacturer protocol to obtain a 100µM stock solution.
    - 25 µM aliquots were prepared and used in the PCR mixture.

2. *Protocol:*

For each sample, the final volume of the PCR reaction mixture was 25 µl and it consisted of, the number in the brackets representing the final concentration of each component in the reaction mixture: 2.5 µl 10xTaq DNA polymerase buffer with $(NH4)_2SO_4$ (×1), 2 µl Magnesium chloride (2mM), 0.6 µl Forward *stx2* primer (0.6 µM), 0.6 µl Reverse *stx2* primer (0.6 µM), 2.5 µl dNTPS (0.2 mM each), 0.125 µl Taq DNA polymerase (0.025U/µl), 2.5 µl extracted DNA (1 µg/ml), and 14.175 µl nuclease free water. A Master Mix was prepared and PCR was carried out on the 3 DNA samples, two positive controls, and a negative control.

The reaction mixtures were placed in a thermal cycler (PCR Sprint Thermal Cycler, Thermo Fisher Scientific, Waltham, MA, USA) which underwent different cycles. The cycling program for the *stx2* primer was as follows: 1 cycle of initial denaturation at 95ºC for 10 minutes, 35 cycles of denaturation at 95ºC for 40 seconds, annealing at 55 ºC for 10 seconds, and elongation at 72 ºC for 45 seconds, and a final cycle of elongation at 72 ºC for 10 minutes.

G. Gel Electrophoresis

1. *Materials needed:*

- 10x TBE (108g Tris Base + 55g Boric acid + 9.3g disodium EDTA added to 1 liter distilled water and then autoclaved) (Amresco, USA)
- Seakem Agarose Powder (Lonza, USA)

- 0.625 mg/ml Ethidium bromide (Amresco, USA)
- 6x Loading dye (Fermentas, USA)
- 100 bp DNA ladder (Fermentas, USA)

2. *Protocol:*

A 1.5% agarose gel was prepared by adding 100 ml of 1x TBE (diluted from 10x TBE) to 1.5 g of Seakem Agarose Powder. The agarose was dissolved by boiling the mixture in the microwave, with regular stirring, until the solution became clear. Two drops of ethidium bromide were added to the gel, after which it was poured into the gel casting tray placed in an electrophoretic chamber with the combs in place. The gel was allowed to cool for approximately 45 minutes until it solidified completely (appeared milky white). 1x TBE was added to the electrophoretic chamber until the gel was submerged. The wells in the gel were loaded with the samples, negative and positive controls, and a 100 bp ladder. The samples were prepared by mixing 2 µl of 6x loading dye with 10 µl of the PCR products. The ladder mixture was prepared by mixing 2 µl of a 100 bp ladder, 2 µl of 6x of loading dye, and 8 µl of 1x TBE. The gel was run at 120 V for approximately 45 minutes. Ultraviolet (UV) transilluminator (Haake buchler Instruments inc., USA) and Olympus digital camera using the Digi-Doc it Program were used for visualizing and photographing the bands respectively.

H. Pulsed Field Gel Electrophoresis (PFGE)

PFGE was carried out on the outbreak and the two pre-outbreak *E. coli* O104:H4 stains to determine their genomic relatedness using the standard operating procedure for Pulsenet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* nonO157 (STEC), *Salmonella* serotypes, *Shigella sonnei*, and *Shigella flexneri*. (164).

1. *Materials needed:*

- 10× TBE (Tris-Boric Acid-EDTA), volume of 500 ml:
  - 60.55 g Tris Base (Amresco, USA)
  - 30.99 g Boric Acid (Amresco, USA)
  - 1.85 g disodium EDTA (Amresco, USA)
  - 500 ml distilled water was added and the mixture was autoclaved
- 0.5 × TBE: 10 × TBE was diluted using autoclaved distilled water
- Cell Lysis Buffer (CLB) (all components were placed in an autoclaved flask), volume of 500 ml:
  - 5 g sarcosyl (N-Lauroylsarcosine sodium salt) (Sigma Chemical Co., St. Louis, MO).
  - 25 ml 1M Tris pH 8 (Amresco, USA)
  - 50 ml 0.5 M EDTA pH 8 (Amresco, USA)
  - Completed with 425 ml double distilled water
- Cell Suspension Buffer (CSB) (all components were placed in an autoclaved flask), volume of 100 ml:
  - 10 ml 1M Tris pH 8 (Amresco, USA)

    - 20 ml 0.5 M EDTA pH 8 (Amresco, USA)
    - Completed with 70 ml autoclaved distilled water
- TE buffer (for washing and plugs preparation), volume of 500 ml:
    - 5 ml 1M Tris pH 8 (Amresco, USA)
    - 1 ml 0.5 M EDTA pH 8 (Amresco, USA)
    - 494 ml autoclaved distilled water
- Seakem Gold (SKG) Agarose for PFGE (Lonza, USA)
- Ladder: BAA 664 (Braendrup) *Salmonella* species
- Autoclaved distilled water
- Nuclease free water
- Proteinase K (20 mg/ml; Thermo Scientific Inc., USA)
- 10 × Tango buffer (Fermentas, USA)
- Incubation buffer mix (1 × Tango buffer), a volume of 2 ml (enough for 10 samples): prepared by mixing 200 µl with 1800 µl nuclease free water and mixed by vortexing
- Restriction mix (for 10 samples), volume of 2 ml:
    - 1755 µl nuclease free water
    - 200 µl Restriction enzyme buffer (10 × Tango buffer)
    - 20 µl Bovine Serum Albumin (BSA) (Amresco, USA)
    - 25 µl XbaI enzyme (Fermentas, USA)
    - Mixed by pipetting the mixture up and down followed by swirling
- Ethidium bromide (Amresco, USA)

2. *Protocol:*

a. Preparation of Seakem Gold Agar Gel for plugs

- The gel (1%) was prepared by mixing 0.5 g of Seakem Gold Agarose in 50 ml TE buffer and the preparation was mixed gently to disperse the agarose.
- The mixture was heated in the microwave (with regular stirring) to dissolve the agarose.
- The gel was incubated at 50 ºC in a water bath for 20 minutes or until ready to use.

b. Preparation of Cell suspension

- In different test tubes labeled with name of the isolates including the ladder, 2 ml CSB was added
- A few colonies from a fresh culture of each isolate including the ladder grown on MacConkey agar plate, were inoculated in their corresponding tubes containing the CSB. The suspension was vortexed to evenly disperse the bacterial cells.
- The concentration of the cell suspension for the samples was adjusted to 2 McFarland, while that of the ladder was adjusted to 2.5 McFarland.

c. Plugs casting

- 400 µl of the adjusted cell suspensions was added to labeled autoclaved 1.5 ml microcentrifuge tubes

- 20 µl of Proteinase K (20 mg/ml) was added to each microcentrifuge tube and the mixture was pipetted up and down.
- The agarose gel was removed from the water bath and 400 µl of the gel was transferred to each microcentrifuge tube and the mixture was pipetted up and down a few times.
- 400 µl of the mixture in each microcentrifuge tube was transferred into separate wells in the plug mold.
- The mold was incubated at 4 ºC for 10 minutes until the plugs solidified.

d. Cell Lysis of agarose plugs

- 5 ml of CLB was transferred to labeled falcon tubes. Subsequently, 25 µl of Proteinase K (20 mg/ml) was added to each falcon tube.
- After solidification, the plugs were removed from the plug mold and dropped into their corresponding falcon tube, making sure they were completely immersed in the buffer.
- The falcon tubes were incubated in a shaker incubator with constant vigorous agitation (150-175 rpm) for 2 hours at 54 ºC.

e. Washing of plugs after cell lysis

- After incubation, the CLB was poured off carefully from each falcon tube and 5 ml of pre-heated (54-55 ºC) autoclaved water was added to each falcon tube. Subsequently, the tubes were incubated in a shaker incubator for 15 minutes at 54

ºC, after which the water was discarded. The washing with autoclaved water was pursued twice.

- After washing the plugs with water, 5 ml preheated TE (54-55 ºC) was added to each falcon tube. The tubes were incubated in a shaker incubator for 15 minutes at 54 ºC, after which the TE was discarded. The washing step with TE was performed 4 times.
- After the last incubation step with TE, the TE was discarded and 5 ml of TE was added to each falcon tube. The falcon tubes were incubated overnight at 4 ºC.

f. Plugs cutting

- On the second day, 200 µl of 1 × Tango buffer (incubation buffer mix) was added to labeled microcentrifuge tubes.
- The falcon tubes containing the plugs were removed from the refrigerator and the plugs were cut into 2 mm thick plug using a blade.
- Each cut plugs was placed in the tango buffer found in the corresponding microcentrifuge tube using a spatula.
- The microcentrifuge tubes were incubated in a walk-in incubator at 37ºC for 15 minutes.

g. Digestion of DNA in agarose plugs

- After incubation, the incubation buffer was discarded from each tube without disturbing the plug, 200 µl of restriction buffer mix was added into each tube, and the tubes were incubated at 37 ºC for 3 hours (XbaI enzyme works at 37 ºC).

h. Preparation of 1% Seakem Gold Agarose

- An hour before the incubation period ended, agarose for gel preparation and for covering the wells were prepared.

i. Agarose for gel preparation

- 1 g of Seakem Gold Agarose was mixed in 100 ml of 0.5 × TBE and the preparation was swirled gently to disperse the agarose.
- The mixture was heated in the microwave (with frequent stirring) until the agarose dissolved.
- The gel was incubated at 56 ºC in a water bath for 20 minutes before pouring.

ii.Agarose preparation for covering the wells

- 0.1 g of Seakem Gold Agarose was mixed in 10 ml 0.5 × TBE and the preparation was mixed gently to disperse the agarose.
- The mixture was heated in the microwave (with regular stirring) to dissolve the agarose.
- The gel was incubated at 56 ºC in a water bath until use.

i. Agarose gel casting

- After cooling, the gel was poured into a gel cast with the corresponding comb.
- The gel was covered with an aluminum foil and left to dry at room temperature for 30-45 minutes.
- After the 3 hours incubation period, the restriction mix was discarded from each microcentrifuge tube without disrupting the plug and 200 µl 0.5 × TBE was added to each tube.
- The microcentrifuge tubes containing the plugs were incubated at room temperature for 10 minutes.

j. Plugs loading into wells

- Each plug was removed from its microcentrifuge tube using a sterile spatula and placed into a well in the gel.
- The wells containing the plugs were completely covered by filling the wells of the gel with 50 µl agarose prepared for well covering. The agarose was left for 3-5 minutes to harden.

k. Running of the gel

- 2200 ml of 0.5 × TBE was poured into the electrophoretic cell (Bio-rad, USA) and the buffer was allowed to cool to 14 ºC (by turning on the cooling module (Bio-rad, USA)). The gel was then removed from the casting mold and placed in the electrophoretic cell.

- The program for Non O157 Shiga toxin producing *Escherichia coli* (STEC) was entered on the CHEF MAPPER^{TM}(Bio-rad, USA) by selecting the following conditions:

  -Auto Algorthim

  50 kb- low MW

  400 kb- high MW

  - Default values were selected except where noted by pressing "Enter"

  - Run Time of 18 hours was entered

  (Default values: Initial switch time= 6.76 s; Final switch time= 35.38s).

- The program was started and the run was carried out overnight.

l. Staining of the PFGE agarose gel

- On the second day, after the run was over, the machine was turned off and the gel was placed in a jar filled with 400 ml distilled water. 8 drops of ethidium bromide were then added.
- The jar was placed on a rocker machine and incubated for 20 minutes.
- After incubation, the ethidium bromide was discarded.

m. Destaining of the PFGE agarose gel

- 500 ml of distilled water was added to the jar containing the gel. The gel was placed on a rocker machine and incubated for 20 minutes. This was repeated 2 more times.

- After discarding the water from the jar for the last time, a picture of the gel was captured using the Gel Doc XR + system Machine (Bio-rad, USA) and the bands were visualized and analyzed with “Quality one” and “Bionumerics” software respectively.

I. RNA extraction

RNA extraction was done using the IIustra RNAspin Mini RNA Isolation Kit (GE healthcare, UK) according to the manufacturer’s specifications for bacterial cells.

1. Materials needed:

- TE buffer (Amresco,USA)
- Lysozyme (stock of 20mg/ml was prepared by adding 30 mg of lysozyme powder to 1.5 ml of nuclease free water. A 0.2 mg/ml solution was obtained by dilution) (USB, USA)
- Buffer RA1 (with kit)
- β-mercaptoethanol
- 70% Ethanol
- Membrane Desalting Buffer (MDB) (with kit)
- DNase I (lyophilized form provided with kit; it was reconstituted by adding 540 µl of nuclease free water. Aliquots of 40 µl were made and stored at -20 ºC)
- DNase Reaction Buffer (with kit)
- Buffer RA2 (with kit)

- Buffer RA3 (with kit)
- RNase free water (with kit)
- Ribolock RNase inhibitor (Fermentas, USA)

2. ***Preparation of the bacterial suspensions for RNA extraction:***

RNA extraction was performed on 16 samples. Three different protocols were followed to prepare the bacterial suspensions for RNA extraction.

a) Protocol 1: To study the effects of rifampicin, gentamicin, or both at the MIC and MBC levels, on the outbreak E.coli O104:H4 strain, RNA extraction was done on the samples shown in Table 1. One ml of the specified dose of the antimicrobial agent was added initially into the tubes corresponding to samples 2-8. However, one ml MH II broth was added to sample 1 tube. Each tube was then inoculated with 1 $\times 10^6$ CFU/ml bacterial suspension (MH II broth was used as a growth medium for the bacteria). The total volume was 2 ml in each tube and all incubations took place at 37ºC.

b) Protocol 2: To determine the effect of sub-inhibitory concentrations of rifampicin and gentamicin on the outbreak *E.coli* O104:H4 strain, another set of RNA extraction was done on the samples shown in Table 2.

   Sub MIC 1 and sub MIC 2 correspond to a two-fold dilution and a four-fold dilution of the MIC of the corresponding antimicrobial agent respectively. A bacterial suspension of 0.5 McFarland in MH II broth was initially prepared. This was subjected to a 4 times dilution, so that the concentration of the 10.24 ml bacterial

suspension was 2.5 $\times 10^7$ CFU/ml. Consequently, the addition of the MH II broth, containing the antimicrobial agents, to the bacterial suspension resulted in a two-fold dilution. Therefore, the final concentration of the bacterial suspension was 1.25 $\times 10^7$ CFU/ml and the total volume in each tube was 20.48 ml. Furthermore, gentamicin stock (5120 µg/ml) was diluted 20 times before being used. All incubations took place in a shaker incubator at 37 ºC for 2 hours with vigorous agitation.

c) Protocol 3: To compare the transcript levels of the toxin gene in the outbreak and the two pre-outbreak *E.coli* O104:H4 strains, RNA extraction was carried on the samples presented in Table 3. Initially, 1 ml MH II broth was added into each tube, after which it was inoculated with 1 $\times 10^6$ CFU/ml bacterial suspension (MH II broth was used as a growth medium for the bacteria) and the total volume in each tubes was 2ml. The samples were incubated overnight at 37 ºC.

3. *RNA extraction protocol:*

The sample "*E.coli* O104:H4 grown alone" in the first preparation of bacterial suspensions was adjusted to have a concentration of 1×10^6 CFU/ml. The bacterial concentration in the rest of the samples of the first bacterial suspension preparation was not adjusted. The samples in the second and third bacterial suspension preparations were adjusted to have a concentration of ~ 0.5 McFarland and 1×10^6 CFU/ml respectively.

a.Cell lysis and Homogenization

- A certain volume of bacterial suspension was taken from each sample: 1 ml from the "*E.coli* O104:H4 grown alone" sample in the first preparation of bacterial suspensions and the samples in the third preparation of bacterial suspension, the whole volume of samples 2-8 in the first preparation of bacterial suspensions, and 1.5 ml of the samples in the second preparation of bacterial suspensions. These were transferred into a microcentrifuge tubes and centrifuged at maximum speed for 15 minutes.
- The bacterial cell pellet of each sample was resuspended in 100 µl TE buffer containing 0.2 mg/ml lysozyme. This was then vigorously vortexed and incubated in a heat block at 37˚C for 10 minutes.
- After incubation, 350µl of RA1 buffer and 3.5 µl of β-mercaptoethanol were added to each mixture. To reduce the viscosity of the suspensions, the samples were vortexed immediately and vigorously.

b.Filtration of lysate

- The mixture in each tube was transferred to a corresponding violet RNAspin Mini filter unit placed in a collection tube; the solution was centrifuged for 1 min at 11,000 x g (12,800 rpm).
- Each filtrate was transferred to a new 1.5 microcentrifuge tube and the RNAspin Mini filter unit was disposed.

c. RNA binding in adjusted conditions

- 350 µl of 70% ethanol was added to each filtrate and the mixture was vortexed for 10 seconds.
- The mixture was pipetted up and down and then transferred to a Blue RNAspin Mini column placed in a collection tube.
- Each sample was centrifuged for 30 sec at 8,000 x g (10,900 rpm) and the column was placed in a new collection tube.

d. Desalt silica membrane and DNA digestion

- 350 µl of Membrane Desalting Buffer (MDB) was added to each column. The samples were then centrifuged for 1min at 11,000 x g (12,800 rpm) to dry the membrane.
- The filtrate was disposed and the column was returned to the same collection tube.
- DNase reaction mixture was prepared by adding 90 µl DNase reaction buffer to 10 µl reconstituted DNase I (per sample) and the solution was mixed by flicking the tube
- 95 µl of the DNase reaction mixture was added directly to the center of the silica membrane (for each sample). The samples were then incubated at room temperature for 30 minutes.

e. Washing and Drying

- 200 µl of buffer RA2 was added to an each RNA spin Mini column, the samples were centrifuged for 1 min at 11,000 x g (12,800 rpm). The column of each sample was placed into a new collection tube.
- 600 µl of buffer RA3 was added to each RNA spin Mini column, the samples were centrifuged for 1 min at 11,000 x g (12,800 rpm). The filtrate was disposed and the column of each sample was placed back into the same collection tube.
- 200 µl of buffer RA3 was added to each RNA spin Mini column, the samples were centrifuged for 2 min at 12,800 rpm (11,000 xg) to. The column of each sample was transferred into a nuclease free 1.5 ml microcentrifuge tube.

f. Elution and Aliquoting

- RNA was eluted in 60 µl RNase free water and the samples were centrifuged at 11,000 x g (12,800 rpm) for 1 min.
- Eluted RNA was immediately placed on ice to prevent potential degradation.
- 1µl Ribolock RNase inhibitor was added to each sample. Aliquots of 20µl were made and stored at -80˚C.

g. RNA concentration determination

- Using a spectrophotomer at an absorbance of 260 nm for nucleic acids, the concentration of the RNA was determined.

J. Reverse Transcription and cDNA synthesis

QuantiTect® Reverse Transcription Kit (Qiagen, Germany) was used to produce cDNA from the extracted RNA according to the manufacturer's specifications. The cDNA was synthesized to be used in RT-qPCR.

1.*Materials needed:*

- Extracted RNA
- gDNA Wipeout Buffer (with kit)
- Quantiscript Reverse Transcriptase containing RNase inhibitor (with kit)
- Quantiscript RT Buffer containing dNTPs and Mg^{2+} (with kit)
- RT primer Mix containing oligo-dT and dissolved in water (with kit)
- RNase free water (with kit)

2. Protocol:

The protocol is usually divided into two major steps: elimination of genomic DNA (gDNA) and reverse transcription. The RNA samples were thawed on ice while the reagents were melted at room temperature. The reagents were then mixed evenly by flicking. They were then centrifuged to collect all the liquids from the edges of the tubes. Finally, the reagents were stored on ice while working on the procedure.

a. <u>Elimination of genomic DNA (gDNA)</u>

Based on the protocol, the RNA used to prepare the cDNA can range between 10 pg and 1 µg. Therefore, 0.1 µg of the RNA was used to prepare the cDNA. In addition, the samples

were diluted using RNase free water. The total volume of RNA and RNase free water was 12 µl. In addition, 2 µl of gDNA wipeout buffer was added making the total reaction volume per sample, 14 µl. The samples were then incubated at 42 ºC for 2 minutes after which they were placed on ice.

b. Reverse Transcription Reaction

The preparation of a master mix containing 1 µl of Quantiscript reverse Transcriptase (per sample), 4 µl of Quantiscript RT buffer (per sample), and 1 µl of RT Primer Mix (per sample) was carried out on ice. Subsequently, 6 µl of the master mix was mixed with the 14 µl mixture (per sample) prepared from the "genomic DNA elimination" step. This yielded a total reaction volume of 20 µl (per sample). Each sample was mixed and placed on ice.

The samples were then placed in a thermal cycler (PCR Sprint Thermal Cycler, Thermo Fisher Scientific, Waltham, MA, USA) to achieve incubation conditions of: 15 minutes at 42 ºC and 3 minutes at 95 ºC. Finally, aliquots of 10 µl of cDNA were prepared and stored at -20 ºC.

K. Real time Polymerase Chain Reaction (RT-qPCR)

The transcription levels of the *stx2* and r*ecA* genes were examined using the QuantiFast™ SYBER® green PCR kit (Qiagen, Germany). The transcription level of the *stx2* gene was detected in all cDNA samples, produced from the corresponding RNA samples (samples from protocol 1: first run of RT-qPCR, samples from protocol 2: second run of RT-qPCR, samples from protocol 3: third run of RT-qPCR).

On the other hand, the transcription level of the *recA* gene was examined in cDNA samples, produced from the corresponding RNA samples from protocol 2 (fourth first run of RT-qPCR). Table 4 summarizes the properties of the genes examined.

1. ***Materials needed:***

- cDNA prepared in section J
- QuantiFast SYBR Green PCR master mix (with kit) including:

  - dNTP mix

  - SYBR Green I

  - HotStar Taq® Plus DNA polymerase which will be activated by the 5 min 95˚C incubation step

  - QuantiFast SYBR Green PCR buffer containing Tris-Cl, KCl, NH_4Cl, $MgCl_2$, and additives
- RNase free water (with kit)
- Real time primers with a concentration of 5 µM (Primers in the lypholized form were reconstituted with a certain amount of 1× TE buffer according to the manufacturer protocol to form 100 µM stock solutions. Aliquots of 50 µM and 25 µM were prepared and stored at -20 ºC diluted; the 5µM primers were diluted from the 50 µM or 25 µM primer solutions and prepared every time when needed).

2. ***Protocol:***

- The samples and reagents were thawed, mixed by vortexing and centrifugation, and kept on ice until use.
- Two separate Master Mixes per RT-qPCR run were prepared for the samples, one for the gene in question (the *recA* or *stx2* gene) and another for the housekeeping gene (the *rpoB* gene). Each master mix included: QuantiFast SYBR Green PCR master mix (10 µl per sample), RNase free water (4 µl per sample), 5 µM Forward primer (2 µl per sample), 5 µM Reverse primer (2 µl per sample); the total volume of master mix per sample was 18 µl.
- A 96 well plate was used for each run and 18 µl of the master mix corresponding to either the gene of inquiry or the housekeeping gene was distributed into the respective wells. This was followed by the addition of 2 µl cDNA samples into their corresponding wells; therefore, each well contained a total volume of 20 µl reaction mixture. Moreover, each sample was run in triplicates for the gene in inquiry and the housekeeping gene.
- The wells were sealed and the plate was centrifuged to make sure no bubbles were present.
- Real time runs were carried out in a Bio-Rad CFX96 Real Time System C1000 Thermal Cycler (Bio-Rad, Germany) and the cycling condition steps for each primer were as follows:
    1. 1 cycle of 95 ºC for 15 minutes (for initial sample denaturation and enzyme activation)
    2. 45 cycles of : 95 ºC for 10 seconds (denaturation)

Ta for 30 seconds (annealing)

72 ºC for 20 seconds (elongation/extension)

3. Melt curve 40 ˚C to 95˚C, increment 0.5 ˚C for 5 seconds (melt curve analysis)
4. 12˚C for 5 minutes

- The transcription levels of the genes of inquiry were calculated, using the Bio Rad CFX manager software, in the samples treated with antimicrobial agents compared to samples without antimicrobial agent, employing the reference gene *rpoB* as a standard (First, second, and fourth run). For the third run, the transcription level of the gene in question was calculated in outbreak strain sample compared with the pre-outbreak strain samples, using the *rpoB* gene as a standard.

L. Reverse Passive Latex Agglutination (RPLA)

RPLA was carried out on two different groups of samples. The first group included the outbreak *E.coli* O104:H4 strain exposed to rifampicin, gentamicin, or both at the MIC and MBC levels. The second group comprised the outbreak *E.coli* O104: H4 strain subjected to two sub MICs of rifampicin and gentamicin. RPLA was performed using VTEC-RPLA kit (OXOID, Japan) in order to compare the differential production of Stx2 in these samples.

1. *Materials needed:*

- Trypticase Soy Broth (TSB)
- U- well Microtitre plate
- Phosphate Buffer Saline (diluent and provided with the kit)

- Sensitized latex VT2, made up of latex suspension sensitized with specific antibodies rabbit IgG against *E.coli* verocytotoxin type 2 (provided with the kit)
- Latex control latex which is a suspension sensitized with non-immune rabbit antibodies (provided with the kit)
- Verotoxin Control VT2 containing dried *E.coli* verocytotoxin type 2 (provided with the kit and reconstituted with 0.5 ml of the diluent)
- Moisture box

2. *Protocol:*

- Bacterial suspension preparation for the first group of samples:
    - For the *E.coli* O104:H4 sample: in a tube containing 2ml TSB, 1×10^6 CFU of the bacteria were inoculated. The tube was incubated on a rotator at 140 oscillations per second (Eberbach Corporation, Michigan, USA) at 37ºC for 20 hours. The final bacterial suspension concentration in this tube was 3 McFarland (cell density of 6×10^8 CFU/ml)
    - For the *E.coli* O104: H4 samples treated with either the MIC of rifampicin, the MBC of rifampicin, the MIC of gentamicin, or the MBC of gentamicin: a starting bacterial concentration of 6×10^8 CFU/ml was inoculated in separate tubes with 2 ml TSB containing the MIC or MBC dose of the corresponding antimicrobial agent. The tubes were placed on a rotator and incubated at 37ºC for 20 hours.

- For the *E.coli* O104: H4 samples treated with either the MIC of rifampicin followed its MBC, the MIC of rifampicin followed by the MBC of gentamicin, or the MIC of gentamicin followed by its MBC: a starting cell density of 6×10^8 CFU/ml was inoculated in separate tubes with 2 ml TSB containing the MIC dose of the respective antimicrobial agent. The samples were placed on a rotator and incubated at 37ºC for 16 hours. After the incubation period, MBC dose of the corresponding antimicrobial agent was added to the samples which were then incubated for another 4 hours (total incubation time 20 hours).

- Bacterial suspension preparation for the second group of samples:
  - For the *E.coli* O104:H4 sample grown alone, 1×10^6 CFU of the bacteria was inoculated in a tube containing 2 ml TSB and this was incubated on a rotator at 37 ºC for 20 hours.
  - For the rest of the samples, 1×10^6 CFU of the bacteria was inoculated in separate tubes with 2 ml TSB containing the sub MIC doses of the respective antimicrobial agents. The tubes were placed on a rotator and incubated at 37ºC for 20 hours.
- On the second day, the bacterial suspensions were centrifuged at 4000 rpm for 20 minutes at 4 ºC and the supernatants were collected.
- 2 columns each containing 8 wells were assigned to each sample on a microtitre plate. 25 µl of PBS, the diluent, was added to each well.

- To the first well of the two columns for each sample, 25 µl of the supernatant was added.
- Two fold dilution of each sample was attained by transferring 25 µl of the sample in the first well into the second well and so on down each column until well seven, from which 25 µl was discarded. Well eight in all columns did not contain any sample.
- 25 µl of the test latex VT2 was added to each well of the first column and 25 µl of latex control was added to each well of the second column.
- A single column at the beginning of the microtitre plate was allocated for the Verotoxin Control VT2. Each well of that column initially contained 25 µl diluent. Subsequently 25 µl of VT2 control was added to the first well and two fold serial dilutions were performed similar to the tested samples. Finally, 25 µl of the test latex VT2 was added to each well of the column.
- The samples were mixed by hand agitation. The plate was covered with its lid and wrapped in damp paper towel. The plate was then placed in a moisture box, an air tight container (to avoid evaporation) and incubated for 20-24 hours at room temperature.
- On the second day, the plate was placed on a black background and a picture of the wells was captured. In addition, each well was observed for agglutination and given a titer value.

M. Protein Extraction

In order to carry out Western Blotting, protein extraction was performed on the outbreak *E.coli* O104: H4 samples exposed to two sub MICs of rifampicin and gentamicin.

1.*Materials needed:*

- DNase, 10 KU (GE healthcare, UK)
- Lysozyme, 20 mg/ml (USB, USA)
- Protease Inhibitor Cocktail (Abcam,UK)
    - Supplied as lyophilized powder.
    - Reconstituted with 250 µl DMSO (Amresco, USA) making a 500 × inhibitor cocktail concentration. The solution was pipetted several times to dissolve the powder.
    - Aliquots of 10 µl were made and stored at -20 ºC.
    - When needed, the protease inhibitor was diluted 500 folds
- Cell Lysis Buffer:
    - 2mM hexahydrate magnesium chloride ($MgCl_2.6H_20$, MW= 203.3; Amresco, USA)
    - 10% glycerol (Sigma Chemical Co., St. Louis, MO)
    - 0.1% Triton X-100 (Bio-Rad, USA)
    - 50mM Tris-HCl buffer, pH 8 (Amresco, USA)
    - 100 µg/ml Lysozyme, 5 units/µl DNase, and × 1 protease inhibitor cocktail were added immediately before use.
- Liquid Nitrogen

- 1 ml syringes

2. *Protocol:*

Protein extraction was done on bacterial suspensions prepared similarly to Protocol 2 in the RNA extraction section (see Table 2).

- After the incubation period of 2 hours, the bacterial suspensions were centrifuged and the pellet in each sample was collected. A certain volume of MH II broth was added to obtain an optical density of 0.6 (3.2 McFarland; obtained from a standard curve).
- The 0.6 OD bacterial suspensions were centrifuged at 5000 rpm for 10 minutes, the pellet of each sample was collected and the supernatant was removed using a pipette.
- To each pellet, 151.8 µl of cell lysis buffer was added and the samples were incubated on ice for 30 minutes.
- After incubation, the samples were thawed in a water bath (37 ºC) and frozen in liquid nitrogen 3 times.
- The samples were passed through 1 ml syringes several times until each mixture was no longer viscous.
- The samples were then centrifuged at 14000 rpm for 20 minutes at 4 ºC.
- The supernatant of each sample was collected and the pellet was discarded.
- Aliquots of 20 µl were prepared and stored at -20 ºC.

N. Bradford Assay

To determine the concentration of the extracted proteins, Bradford assay was performed.

1. ***Materials needed:***

- Extracted proteins
- Bovine serum albumin (BSA) (1 µg/ml)
- Bradford Reagent (Biorad, USA)
- Nuclease free distilled water (Amresco, USA)
- 1ml cuvettes

2. ***Protocol:***

- The proteins were thawed and placed on ice immediately to preserve the protein.
- Bovine serum albumin was used as the protein standard and 9 microcentrifuge tubes were prepared as shown in Table 5 for the standard curve (total volume per tube was 1 ml).
- For each extracted protein sample (unknown concentration), a separate microcentrifuge tube was prepared and it contained: 798 µl of distilled water, 200 µl Bradford reagent, and 2 µl of the extracted protein.
- The solutions are mixed vigorously by vortexing for 10 seconds. The mixtures were then incubated at room temperature for 10 minutes.
- Using a spectrophotometer, the absorbance of the standard curve samples (known concentration) were initially measured followed by that of the extracted protein

(unknown concentration) at a wavelength of 595 nm. The initial concentration of the extracted protein was calculated using the following equation: Initial Concentration of extracted protein (µg/µl)= (Concentration of extracted protein measured using spectrophotometer × 1000 µl)/2µl.

O. Western Blotting

In order to study the expression of the SOS response repressor, LexA, western blotting was carried out at two sub MICs of rifampicin and gentamicin. The experiment is made up of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS- PAGE) and western blotting.

1. ***Materials needed:***

- SDS-PAGE gel electrophoresis setup (Biorad, USA)
- Lower and upper gel preparation
    - 1.5 M Tris, pH 8.8 (lower gel; Biorad,USA)
    - 1.0 M Tris, pH 6.8 (upper gel; Biorad,USA)
    - 30% Acrylamide mix consisting of 29.2% Acrylamide and 0.8% N,N'-methylene-bisacrylamide (Biorad,USA).
    - 10% SDS: 10 g SDS in 100 ml autoclaved distilled water; dissolved by mixing gently (Biorad,USA)
    - 10% Ammonium persulphate (APS): 1g in 10 ml autoclaved distilled water (Biorad,USA)

    - N,N,N’,N’-tetramethyl-ethane-1,2-diamine (TEMED) (Biorad,USA)
    - n-butanol
    - Autoclaved distilled water
- 5× SDS loading dye
- Dithiothreitol (1 M): 1.5 g in 10 ml autoclaved distilled water. Aliquots were made and stored at -20 ºC.
- Broad Range Markers: sc-2361 Protein ladder (Santa Cruz Biotechnology Inc., USA)
- 5 × Running buffer (5 × Tris glycine electrophoresis buffer): Volume of 1 Liter
    - 15.1 g Tris base
    - 94 g Glycine
    - 50 ml 10% SDS
    - 950 ml autoclaved distilled water
- 1× Running buffer: 5 × Running buffer was diluted using autoclaved distilled water
- Amersham Hybond-P PVDF Membrane (GE Healthcare, UK)
- Filter paper and sponges
- Transfer membrane setup (Biorad, USA)
- 10× Transfer buffer Saline (TSB): Volume of 1 liter
    - 80 g Sodium chloride
    - 30 g Tris base
    - 2 g Potassium chloride

    - Initially 800 ml autoclaved distilled water was added, pH was adjusted to 8 using hydrochloric acid, and the rest of the 1 liter volume was completed with autoclaved distilled water

- 1 × TSB: 10 × TSB was diluted using autoclaved distilled water
- TBT (Transfer Buffer Tween): Volume of 1 liter
    - 100 ml 10 × TSB
    - 2 ml Tween-20
    - Volume was completed with autoclaved distilled water
- Methanol
- Blocking buffer: 5% regile' fat free milk in 1 × TBT (2 g in 40 ml TBT)
- 10 × Transfer Buffer (TB, 5 × Tris-Glycine electrophoresis buffer but does not contain SDS), Volume of 1 liter:
    - 15.1 g Tris base
    - 94 g Glycine
    - 1 liter of autoclaved distilled water
- 1 × Transfer Buffer
    - 100 ml 10 × Transfer Buffer
    - 900 ml autoclaved distilled water
    - 100 ml of mixture was removed and replaced by 100 ml methanol
- Amersham ECL Western blotting detection reagents and analysis system (GE Healthcare, UK)

- Primary Antibody: Anti-LexA (2-12): sc-7544 (Santa Cruz Biotechnology Inc., USA)
    - Mouse monoclonal antibody IgG
    - Molecular weight of LexA is 23 kDa
- Secondary Antibody: Anti-mouse IgG-HRP: RPN4201, derived from sheep (GE healthcare, UK)
- Primary Antibody: Anti-L9 ribosomal protein (Gift from Dr. Isabella Moll, Max F. Perutz Laboratories, Vienna)
    - 30mg lyophilized antibody reconstituted in 300 µl 1x TBS.
    - Goat derived
    - Molecular weight is 22 kDa
- Secondary Antibody: Anti-goat IgG-HRP: sc-2768, derived from rabbit (Santa Cruz Biotechnology Inc., USA)
- Stripping Buffer:
    - 10 ml 10% SDS
    - 6.25 ml 0.5 M Tris (pH= 6.8)
    - 33.75 ml autoclaved distilled water
    - 4 µl β-mercaptoethanol
- Autoradiography cassette
- AGFA film

2. *Protocol:*

- Lower gel (Resolving gel) was prepared as follows (12.5% per 25 ml):
    - 8.2 ml Distilled water
    - 6.3 ml 1.5 M Tris (pH= 8.8)
    - 10 ml 30 % Acrylamide mix
    - 0.25 ml 10% SDS
    - 0.25 ml 10% APS
    - 0.01 ml TEMED

APS and TEMED were added at the end since they are polymerizing agents. n-butanol was added on top of the resolving gel to prevent oxidation, insulate it from the surrounding environment, and linearize the gel. After the lower gel solidified, the upper gel was added.

- Upper gel (Stacking gel) was prepared as follows (5% per 5ml):
    - 3.4 ml Distilled water
    - 0.63 ml 1.0 M Tris (pH= 6.8)
    - 0.83 ml 30 % Acrylamide mix
    - 0.05 ml 10% SDS
    - 0.05 ml 10% APS
    - 0.005 ml TEMED

Before adding the upper gel preparation, the n-butanol was discarded by pouring it off. APS and TEMED were added at the end. Combs were added to form the wells and the gel was incubated at room temperature until it solidified.

- Protein samples were prepared for loading. To each 20 µl samples, 5 µl 5 × SDS loading dye containing DTT was added, so that total volume to be loaded was 25µl

(1 µl of DTT was added to the total volume of 5 × SDS loading dye needed). The mixtures were spun for few seconds.

- To denature the proteins, the samples (protein + dye) were boiled for 3 minutes.
- After the SDS-PAGE gel electrophoresis was assembled, 1 × running buffer was added to the chamber, 5 µl protein ladder was loaded into the first well, and the protein samples were added into their corresponding wells.
- The gel was run initially at 120 V until the dye crossed the upper gel. The voltage was then increased to 150 V until the dye reached the end of the gel.
- Polyvinylidine fluoride (PVDF) membrane was cut (size was cut approximately equal to that of the gel), after which it was activated by dipping it in methanol (handled with care to prevent fingerprinting). In addition, 4 filter papers and 2 sponges were cut and immersed in 1× transfer buffer.
- The materials were placed in the following order on the transfer casing: black side of the casing /Sponge/2 filter papers/Lower Gel (the upper gel was discarded)/membrane/2 filter papers/Sponge /white side of the casing. This was then sealed to produce the “Sandwich”; the sandwich was covered in transfer buffer.
- The transfer apparatus was assembled, the sandwich was placed in the transfer chamber, and an ice pack was added at the back of the compartment (to prevent the gel from melting). A magnetic bar was placed at the bottom of the compartment, the stirrer was run, the voltage was set at 100 V, and the transfer was carried out for 2 hours.

- Following the transfer step, the membrane was removed using forceps (the gel was discarded) and soaked in 1× TBT. The membrane was immersed in methanol and then dried by waving it. This step was repeated two more times.
- The membrane was soaked in blocking buffer and incubated for 1 hour at room temperature on a shaker. After the time period, the blocking buffer was discarded and the membrane was rinsed with 1× TBT.
- Primary antibody anti-LexA was prepared as follows to achieve a 1000 fold dilution: 10 µl of anti- LexA was added to 10 ml 1% milk (1 g in 10 ml 1× TBT). After the prepared primary antibody was added to the membrane, it was incubated at room temperature on a shaker for 2 hours.
- After incubation, the primary antibody was returned back to its falcon tube and the membrane was washed with 1× TBT on a shaker for 5 minutes (to remove the residual primary antibody). This step was repeated three times.
- Secondary antibody anti-mouse was prepared as follows to attain a 5000 fold dilution: 2 µl anti-mouse antibody was added to 10 ml 1× TBT. The secondary antibody was added to the membrane after which the membrane was incubated at room temperature on a shaker for an hour. The primary antibody was then returned back to its falcon tube.
- The membrane was washed with 1× TBT on a shaker for 5 minutes. This step was repeated three times.
- In a falcon tube, 750 µl of reagent A and 750 µl of reagent B = ECL were added and mixed. The preparation was added to the membrane drop by drop (ensuring that

the mixture was spread on the whole membrane) and left for 2 minutes. The membrane was dried on paper towel, covered with nylon, and placed on a cassette to carry out autoradiography for 30 seconds initially, followed by 1 minute, then 3 minutes, and finally 5 minutes.

- The membrane was stripped to add anti-L9 in order to ensure that the loading was equal. The membrane (protein side inside) was placed in a falcon tube containing stripping buffer and the falcon tube was placed in a rotating heater preheated to 51 ºC for 30 minutes.
- The membrane was initially washed for 5 minutes on a shaker in 1× TBT after which it was washed with blocking buffer (5% milk) for 45 minutes on a shaker. After the incubation period, the blocking buffer was discarded.
- The primary anti-L9 antibody was prepared as follows: 5 µl anti-L9 was added to 10 ml 1% milk (0.1g in 10 ml 1× TBT); a 1: 2000 dilution. The prepared primary antibody was added to the membrane (placed on a shaker). This was then incubated overnight at 4 ºC in a cold room.
- On the second day, the primary antibody was returned back to its falcon tube and the membrane was washed with 1× TBT on a shaker for 5 minutes. This was repeated 3 times.
- Secondary anti-goat antibody was prepared as follows: 2 µl anti-goat antibody was added to 10 ml 1× TBT; a 1: 5000 dilution. The prepared antibody was added to the membrane which was incubated on a shaker for 1 hour.

- After incubation, the secondary antibody was returned back to its falcon tube and the membrane was washed with 1× TBT on a shaker for 5 minutes. This was repeated 3 times.
- In a falcon tube, 750 µl of reagent A and 750 µl of reagent B = ECL were added and mixed. The preparation was added to the membrane drop by drop (ensuring that the mixture was spread on the whole membrane) and left for 2 minutes. The membrane was dried on paper towel, covered with nylon and placed on a cassette to carry out autoradiography for 30 seconds initially, followed by 1 minute, then 3 minutes, and finally 5 minutes.

P. Determination of LD_{50} of the outreak *E.coli* O104:H4 strain in BALB/c Mice

Adult, Female, 4-8 weeks old mice were obtained from the Animal Care Facility at the American University of Beirut (AUB). The mice were cared for and handled according to "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources Committee on Life Sciences Nation Research Council (165). In addition, the mice were allowed to consume food and fluids as much as desired without restrictions. The experiments on the mice were approved by The Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut.

Although the preferable route of mouse inoculation is oral, intraperitoneal infection was used since BALB/c mice are resistant to Shiga toxin producing *E.coli* infection via oral route.

The LD_{50} of *E.coli* O104:H4 in BALB/c Mice was identified using the method described by Nowotny (166). A total of 20 mice were distributed into 5 groups. The groups received incremental doses of *E. coli* O104:H4. These doses include: 10^4, 10^5, 10^6, 10^7,and 10^8 CFU suspended in 0.2 ml TSB. The mice were monitored for survival for a period of 7 days. The LD_{50} was determined using the formula: Log LD_{50} = log (highest dose tested) + (log D)[(1/2) – (ΣR/N)] where D is the fold difference between successive doses, ΣR is the total number of dead mice after 7 days of monitoring and N is the number of animals per group.

Q. Treatment of *E.coli* O104:H4 infected BALB/c mice using antimicrobial agents

1. *Mice groups*

80 mice, 50 females and 30 males, were equally divided into 10 groups so that each group contained: 5 females and 3 males. The mice were 4-8 weeks old and their weight ranged between 19-41 grams (females: 19-33g, males: 31-42 g). The injections were prepared in a way such that the maximum total volume to be administered per mouse would not exceed 0.5 ml. The injection volumes were: 0.2 ml TSB containing three times the LD_{50} of the outbreak *E.coli* O104:H4 strain for the bacterial injections, 0.1ml TSB for the negative control injections, 0.12 ml rifampicin and 0.18 ml gentamicin for the antimicrobial agents injections. In addition, all the injections were administered intraperitoneally. The first injections were administered at the beginning of the experiment (hour 0), the second injections were given 1 hour later (hour 1), and the third injections were administered 16 hours after the second injections (hour 17). The mice were monitored for a period of two

weeks for weight loss and survival. Table 6 shows the distribution of mice into various groups with different treatment regimens.

2. ***Preparation of bacterial suspension injections***

The bacterial dose that was administered for infection of the mice was three times the LD_{50} of *E.coli* O104:H4. To prepare the bacterial injections, initially several colonies from a fresh culture of *E.coli* O104:H4 grown on MacConkey agar plate were inoculated in 3 ml TSB and incubated overnight at 37 º C. On the second day, the concentration of the bacterial suspension was determined using a turbidimeter (CFU/ml). The suspesnsion was centrifuged at 3500 rpm for 20 minutes. The supernatant was discarded, the pellet was re-suspended in a certain volume of TSB and injections of 0.2 ml were prepared.

3. ***Preparation of antibiotics injections***

The therapeutically relevant *in vivo* MIC equivalent dose of the antimicrobial agents used (rifampicin and gentamicin) was extrapolated from their *in vitro* MIC according to the following formula: Antimicrobial agent *in vivo* MIC dose (µg) = [Antimicrobial agent *in vitro* MIC (µg/µl) x *in vitro* MIC broth volume (µl) x *E. coli* O104:H4 CFU administered *in vivo*] / *E. coli* O104:H4 CFU per *in vitro* MIC reaction. Similarly, the therapeutically relevant *in vivo* MBC equivalent dose of the antimicrobial agents used was extrapolated from their *in vitro* MBC according to the following formula: Antimicrobial agent *in vivo* MBC dose (µg) = [Antimicrobial agent *in vitro* MBC (µg/ µl) x *in vitro* MBC broth volume (µl) x *E. coli* O104:H4 CFU administered *in vivo*] / *E. coli* O104:H4 CFU per *in vitro* MBC reaction.

The antibiotics stock solutions were prepared according to the protocol described in section B and the working solutions were prepared using sterile distilled water. Concerning rifampicin, mice given the MIC dose were injected with 540.8 µg rifampicin in 0.12 ml water and methanol (9:1). On the other hand, the MBC dose administered to the mice contained 1018.18 µg rifampicin in 0.12 ml water and methanol (9:1). For gentamicin, mice that received the MIC dose were injected with 33.8µg gentamicin in 0.18 ml water, while those that were given the MBC dose were injected with 135.2 µg gentamicin in 0.18 ml water.

4. *Mice Dissection and API*

During the monitoring period, 1 dead mouse from each group (except the negative control and drug control group) was dissected. Blood was collected and then cultured on MacConkey agar plates to check for the presence of *E.coli* O104:H4. Colonies that grew on the agar plates were identified to the species level using API20E kit (Biomérieux, France). This allowed for the verification of the cause of death. After the monitoring period, 1 mouse from each of the negative control group and drug control group was euthanized, dissected, after which blood was collected and cultured on MacConkey agar plate to examine if *E.coli* O104:H4 was present.

R.Statistical Analysis:

Statistical analysis was done for the transcription levels of the *stx2* and *recA* genes using unpaired student t- test. All p-values ≤ 0.05 were considered to be statistically significant.

CHAPTER IV

RESULTS

The MIC and MBC of rifampicin for the outbreak *E.coli* O104:H4 strain were 16 µg/ml and 32 µg/ml respectively. On the other hand, the MIC and MBC of gentamicin were 1 µg/ml and 4 µg/ml respectively. Two sub-inhibitory concentrations of the antimicrobial agents were employed during the study, where sub-MIC 1 is a two-fold dilution of the MIC and sub-MIC 2 is a four-fold dilution of the MIC. The sub MIC 1 of rifampicin and that of gentamicin were 8 µg/ml and 0.5 µg/ml respectively, whereas the sub MIC 2 of rifampicin and that of gentamicin were 4 µg/ml and 0.25 µg/ml respectively.

The presence of the *stx2* gene was detected in the outbreak strain and the two pre-outbreak strains by PCR (Figure 1).

The DNA banding patterns revealed by PFGE analysis of the outbreak strain and the pre-outbreak strains 2009EL-2050 and 2009EL-2071 indicated genomic relatedness of 86.4% and 83.7% respectively. This denotes that the outbreak and pre-outbreak strains are closely linked (Figure 2 and 3).

The Real time Polymearse Chain Reaction (RT-qPCR) analysis indicated that the *stx2* gene was expressed in the outbreak and pre-outbreak strains. In addition, the *stx2* gene transcript levels in the outbreak strain was 1.41 times (p-value: 0.0396) that of the 2009 EL-2050 pre-outbreak strain and 1.75 times (p-value: 0.0384) that of the 2009 EL-2071 pre-outbreak strain (Figure 4). Moreover, RT-qPCR analysis showed that the treatment of the outbreak strain with the MIC of rifampicin resulted in a marked decrease (80%) in the transcript levels of the *stx2* gene in comparison to the control (*E.coli* O104:H4 without

antimicrobial agent) (p-value: 0.0448). On the other hand, complete inhibition of the *stx2* gene transcript levels was observed upon treatment of the outbreak strain with the MBC of rifampicin (p-value: 0.009), the MBC of gentamicin (p value: 0.0230), the MIC of rifampicin followed by its MBC (p-value: 0.0137), the MIC of rifampicin followed by the MBC of gentamicin (p-value: 0.0138) and the MIC of gentamicin followed by its MBC (p-value: 0.009). The least inhibition of the *stx2* gene transcript level in comparison to the control was observed in the sample of the outbreak *E.coli* O104:H4 treated with the MIC of gentamicin (52% decrease) (p-value: 0.0128) (Figure 5).

RT-qPCR results showed that the outbreak *E.coli* O104:H4 samples exposed to the sub MIC 1 and 2 of rifampicin resulted in a lower *recA* gene transcript level (p-value: 0.0369 and 0.3881 respectively) and a higher *stx2* gene transcript level (p- value: 0.8993 and 0.5674 respectively) in comparison to the control (*E.coli* O104:H4 without antimicrobial agent). On the other hand, gentamicin at the sub MIC 1 and 2 concentrations, resulted in higher *recA* (p- value: 0.0859 and 0.0744 respectively) and *stx2* gene transcript levels (p- value: 0.2434 and 0.0163 respectively) when compared with the control (Figure 6).

Reverse passive latex agglutination (RPLA) results indicated that the treatment of the outbreak strain with rifampicin, gentamicin, or both at the MIC and MBC levels, resulted in either a 4 fold decrease (the samples treated with the MIC of rifampicin, the MBC of gentamicin, the MIC of rifampicin followed by its MBC, and the MIC of rifampicin followed by the MBC of gentamicin) or 2 fold decrease (the samples treated with the MBC of rifampicin, the MIC of gentamicin, and the MBC of gentamicin followed by its MBC) in Stx2 release in comparison with the control (*E.coli* O104:H4 without

antimicrobial agent) (Figure 7 ,8, and 9). On the other hand, treatment of the outbreak strain with the sub MIC 1 and 2 of rifampicin resulted in an increase in Stx2 release when compared to the control (2 fold and 4 fold respectively). Gentamicin at the sub MIC 1 level produced an equal release of Stx2 in comparison to the control (*E.coli* O104:H4 without antimicrobial agent), whereas gentamicin at the sub MIC 2 level resulted in a two fold increase in the release of Stx2 when compared to the control (Figure 10 and 11).

The results of a western blot assessing the relative levels of Lex A in the outbreak strain subjected to two sub MIC levels of rifampicin and gentamicin showed that the expression of this protein is completely inhibited at both sub MICs of gentamicin (Figure 12); this indicates that the SOS response was de-repressed upon such a treatment. In addition, rifampicin at the sub MIC 1 level led to a low expression of the LexA protein when compared to the non-antimicrobial agent treated control. However, the sub MIC 2 of rifampicin resulted in a higher expression of the LexA protein than the control (Figure 12 and 13). Figure 14 shows the differences in the expression of the LexA protein and the *recA* gene in the outbreak strain subjected to two sub MICs of rifampicin and gentamicin.

The LD_{50} of the outbreak *E.coli* O104:H4 strain was determined and was found out to be 5.16×10^6 CFU. The $3\times$ LD_{50} dose was then used for assessing the effect of antimicrobial treatment in infected mice. The details about the average weight loss and survival during the 2 weeks period of BALB/c mice infected with $3\times$ LD_{50} of *E.coli* O104:H4 and exposed to different combinations and doses of rifampicin and gentamicin are shown in Table 7. All mice that did not receive the bacterial injections, those present in the negative control group and the drug control group survived the 2 weeks observing period. On the other hand, all mice in the untreated group, infected with *E.coli* O104:H4

only, were dead 5 days after the infection (Figure 15). During the monitoring period, a 50% survival rate (4 out of 8 survived), the highest survival percentage, was observed in the group of infected mice that received the MBC of gentamicin. Three mice of the original 8 mice in the infected groups that received the MIC of rifampicin, the MBC of gentamicin, the MIC of rifampicin followed by the MBC of gentamicin, and the MIC of gentamicin followed by its MBC remained alive during the observing period, corresponding to a 37.5% survival rate. The lowest survival rate (25%), corresponding to the survival of 2 out of 8 mice, was observed in the infected group that received the MIC of rifampicin and the group that received the MIC of rifampicin followed by its MBC (Figure 16).

During the monitoring period, the average weight of the mice in the negative control group increased, while that of the mice in the drug control group remained fairly stable. The mice in the groups that were injected with the outbreak isolate bacterial injection (treated and untreated with antimicrobial agents), started losing weight 1 day after the beginning of the experiment. The survivors in the two groups that were treated with the MIC of gentamicin and the MBC of gentamicin began to gradually regain weight 5 days post infection. On the hand, the average weight of the mice that survived in the group that received the MIC of rifampicin, the group that received the MIC of gentamicin followed its MBC, and the group that received the MIC of rifampicin followed by its MBC started increasing 7 days post infection. The average weight of the mice that remained alive in the group treated with the MIC of rifampicin followed by the MBC of gentamicin and the group treated with the MBC of rifampicin began to increase 4 and 8 days post infection respectively. The average weight change per group monitored for two weeks is presented in Figure 17.

The API results showed that the colonies that grew on the agar plates from the blood of all dead dissected mice infected with the bacteria and then treated with a certain dose or combination of the antimicrobial agents were *Escherichia coli* (166). No bacterial growth was observed on agar plates from the blood of dissected mice in the negative control and drugs control group.

Table 1: *E.coli* O104:H4 outbreak strain D3774/C22711 samples subjected to different antimicrobial treatments.

Sample	
1	*E.coli* O104:H4 grown alone for 18 hours
2	*E.coli* O104:H4 incubated with **MIC dose of Rifampicin** for 18 hours
3	*E.coli* O104:H4 incubated with **MIC dose of Gentamicin** for 18 hours
4	*E.coli* O104:H4 grown with **MBC dose of Rifampicin** for 18 hours
5	*E.coli* O104:H4 incubated with **MBC dose of Gentamicin** for 18 hours
6	*E.coli* O104:H4 grown with **MIC dose of Rifampicin** for 18 hours, cells were centrifuged, supernatant was discarded and the pellet was re-suspended with **MBC dose of Rifampicin** and incubated for 4 more hours
7	*E.coli* O104:H4 incubated with **MIC dose of Rifampicin** for 18 hours, cells were centrifuged, supernatant was discarded and the pellet was re-suspended with **MBC dose of Gentamicin** and incubated for 4 more hours
8	*E.coli* O104:H4 grown with **MIC dose of Gentamicin** for 18 hours, cells were centrifuged, supernatant was discarded and the pellet was re-suspended with **MBC dose of Gentamicin** and incubated for 4 more hours

Table 2:. *E.coli* O104:H4 outbreak strain D3774/C22711 samples subjected to sub-inhibitory concentrations of rifampicin and gentamicin.

Antimicrobial agent/concentration	Volume of MH broth (ml)	Volume of bacterial suspension (ml)	Volume of antimicrobial agent (µl)
Rifampicin /sub MIC 1	10.224	10.24	16
Rifampicin /sub MIC 2	10.232	10.24	8
Gentamicin/sub MIC 1	10.20	10.24	40
Gentamicin/sub MIC 1	10.22	10.24	20
-/-	10.24	10.24	0

Table 3: *E.coli* O104:H4 samples for comparing the transcript levels of the toxin gene in the outbreak and pre-outbreak strains.

Sample
The outbreak *E.coli* O104:H4 (D3774/C22711) strain grown alone for 16 hours
The pre-outbreak *E.coli* O104:H4 (2009EL-2050) strain grown alone for 16 hours
The outbreak *E.coli* O104:H4 (2009EL-2071) strain grown alone for 16 hours

Table 4:. RT-qPCR Primers for the *recA*, *stx2*, and *rpoB* genes: Sequence, Size, Amplicon size, annealing temperature (Ta), and reference. Jinneman et al., 2003 (167), Shilpakala et al., 2009 (168)

Gene	Primer Type	Primer Sequence 5' to 3'	Primer Size	Amplicon Size	Ta	Reference
stx2	Fwd Rev	GAT GTT TAT GGC GGT TTT ATT TGC TGG AAA ACT CAA TTT TAC CTT TAG CA	24bp 26bp	83bp	61	Jinneman et al., 2003
rec A	Fwd Rev	ATA TCG ACG CCC AGT TTA CG GTT CCA TGG ATG TGG AAA CC	20 bp 20 bp	235 bp	56	Shilpakala et al., 2009
***rpoB* (reference gene)**	Fwd Rev	TCG AAA CGC CTG AAG GTC TTG GAG TTC GCC TGA GC	18bp 17bp	184bp	52	Designed by Ahmad Sabra

Table 5:. Samples for Bradford Assay. V= Volume, BSA= Bovine Serum Albumin

Samples	V of Distilled water(µl)	V of BSA (µl)	V of Bradford reagent (µl)	BSA concentration (µg/µl)
S1	800	-	200	Blank
S2	800	-	200	-
S3	798	2	200	0.002
S4	796	4	200	0.004
S5	794	6	200	0.006
S6	792	8	200	0.008
S7	790	10	200	0.010
S8	788	12	200	0.012
S9	786	14	200	0.014

Table 6: Mice groups and treatment regimens on the outbreak *E.coli* O104:H4 strain D3774/C22711. TSB: Trpticase Soy Broth

Mice group	First injection (hour 0)	Second injection (hour 1)	Third injection (hour 17)
Group 1 (negative control)	TSB	TSB	TSB
Group 2	3× LD_{50} of *E.coli* O104:H4	-	-
Group 3	3× LD_{50} of *E.coli* O104:H4	MIC rifampicin	-
Group 4	3× LD_{50} of *E.coli* O104:H4	MBC rifampicin	-
Group 5	3× LD_{50} of *E.coli* O104:H4	MIC gentamicin	-
Group 6	3× LD_{50} of *E.coli* O104:H4	MBC gentamicin	-
Group 7	3× LD_{50} of *E.coli* O104:H4	MIC rifampicin	MBC rifampicin
Group 8	3× LD_{50} of *E.coli* O104:H4	MIC rifampicin	MBC gentamicin
Group 9	3× LD_{50} of *E.coli* O104:H4	MIC gentamicin	MBC gentamicin
Group 10 (drug control)	TSB	MIC rifampicin	MBC gentamicin

Table 7: Average weight and survival of each mice group during the 14 days monitoring period. (Group numbers corresponds to those in Table 6)

Groups		Days Post Infection														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group 10	Average Weight	33.3	32.3	32.5	32.8	33	33.4	33.4	33.4	33.3	33.9	33.5	33.8	33.6	33.6	33.9
	Survivors	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Group 9	Average Weight	31	29.1	26.9	25.7	25.8	25.8	26	25.8	28.3	29	29.7	30	30.7	30.7	30.7
	Survivors	8	8	7	7	4	4	4	4	3	3	3	3	3	3	3
Group 8	Average Weight	30.4	28.1	27.7	26.1	28.7	29	29.3	29.3	30	31	32	33	34	33.3	33.7
	Survivors	8	8	7	7	3	3	3	3	3	3	3	3	3	3	3
Group 7	Average Weight	33.6	31.5	29.4	27.5	26	26	25.8	30	30.5	31	30.5	30.5	31	31.5	31
	Survivors	8	8	8	8	7	5	5	2	2	2	2	2	2	2	2
Group 6	Average Weight	34	32	31	30.1	28.7	29.5	29.8	30.8	32	32.8	33.3	34.5	34.5	33.8	34.5
	Survivors	8	8	8	8	6	4	4	4	4	4	4	4	4	4	4
Group 5	Average Weight	27.4	25.1	24.5	23.4	21	24	25	23.5	25.3	26	27	28	28.5	28.8	29.5
	Survivors	8	8	8	8	5	3	3	3	3	3	3	3	3	3	3
Group 4	Average Weight	28.9	26.8	25.5	23.9	23.1	23	21.5	20	21	21.3	22	22	23	22.7	23.3
	Survivors	8	8	8	8	7	5	5	4	3	3	3	3	3	3	3
Group 3	Average Weight	31	27.5	26.6	25.4	25.7	23	24.5	26	27.5	28.5	29	29	29	29.5	30
	Survivors	8	8	8	8	3	2	2	2	2	2	2	2	2	2	2
Group 2	Average Weight	31.8	29.5	27.4	25.6	26	0	0	0	0	0	0	0	0	0	0
	Survivors	8	8	8	8	2	0	0	0	0	0	0	0	0	0	0
Group 1	Average Weight	29.1	29.8	30.4	30.4	30.4	31.1	31.3	31.3	31.1	31.8	32	32.1	31.9	32	32.3
	Survivors	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Groups		Days Post infection														

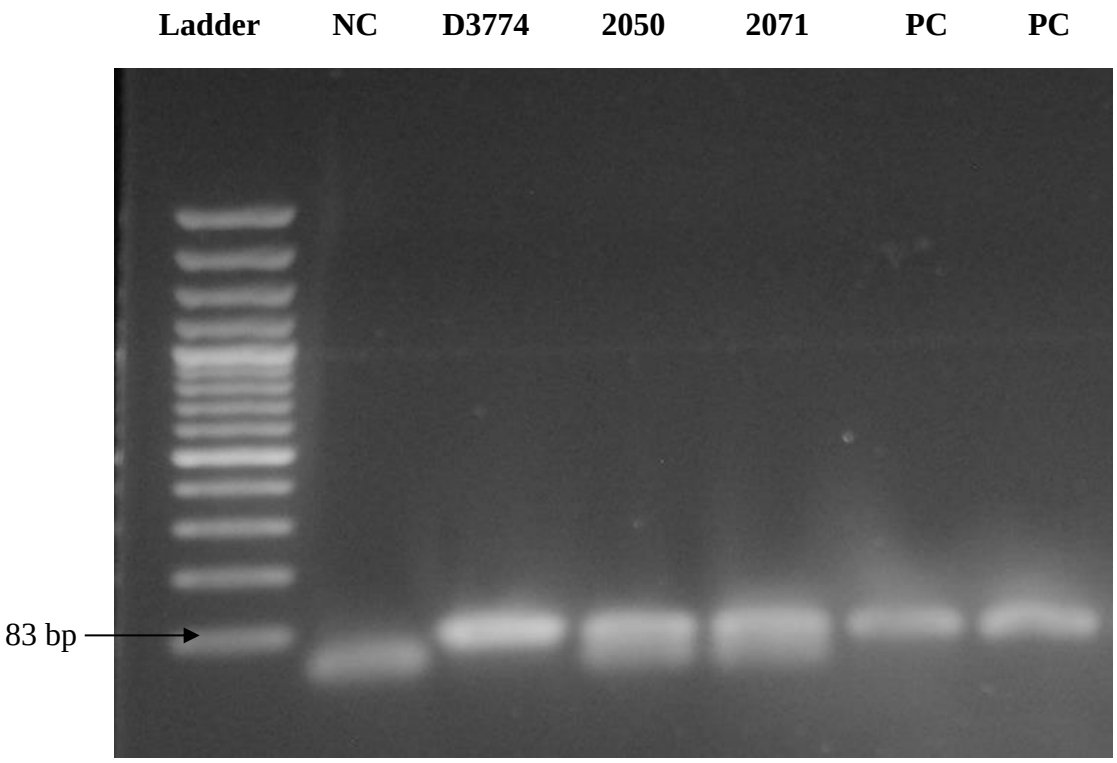


Figure 1: PCR results for the detection of the *stx2* gene, in the outbreak *E.coli* O104:H4 strain D3774/C22711 and the two pre-outbreak *E.coli* O104:H4 strains 2009EL-2050 and 2009EL-2071 (NC: negative control; PC: positive control).

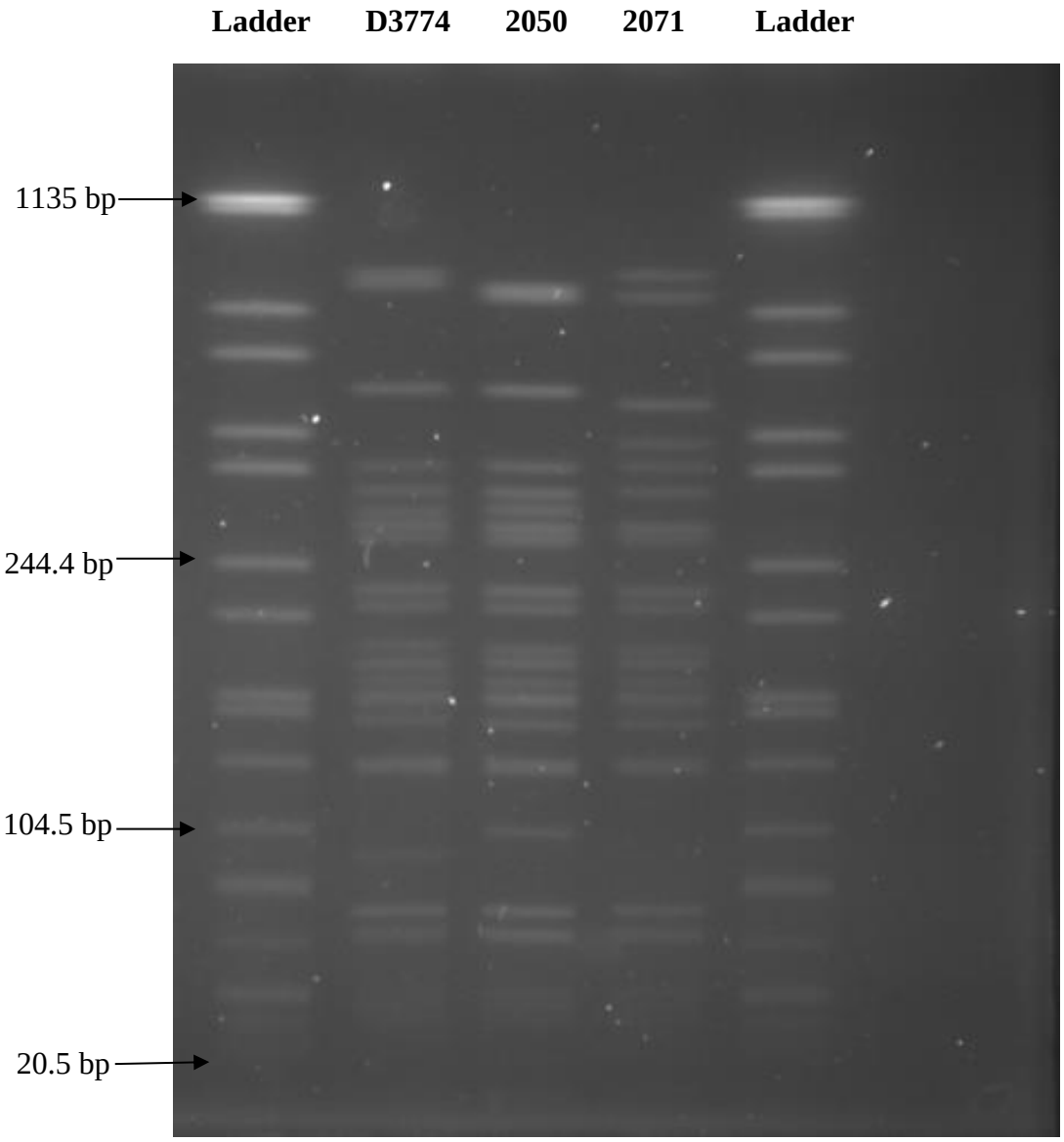


Figure 2: PFGE gel analysis for the outbreak *E.coli* O104:H4 strain D3774/C22711 and the two pre-outbreak *E.coli* O104:H4 strains 2009EL -2050 and 2009EL-2071.

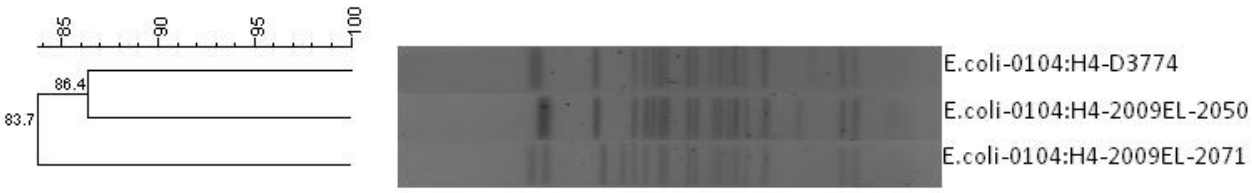


Figure 3: Dendrogram of the PFGE gel for the outbreak *E.coli* O104:H4 strain D3774/C22711 and the two pre-outbreak *E.coli* O104:H4 strains 2009EL -2050 and 2009EL-2071.

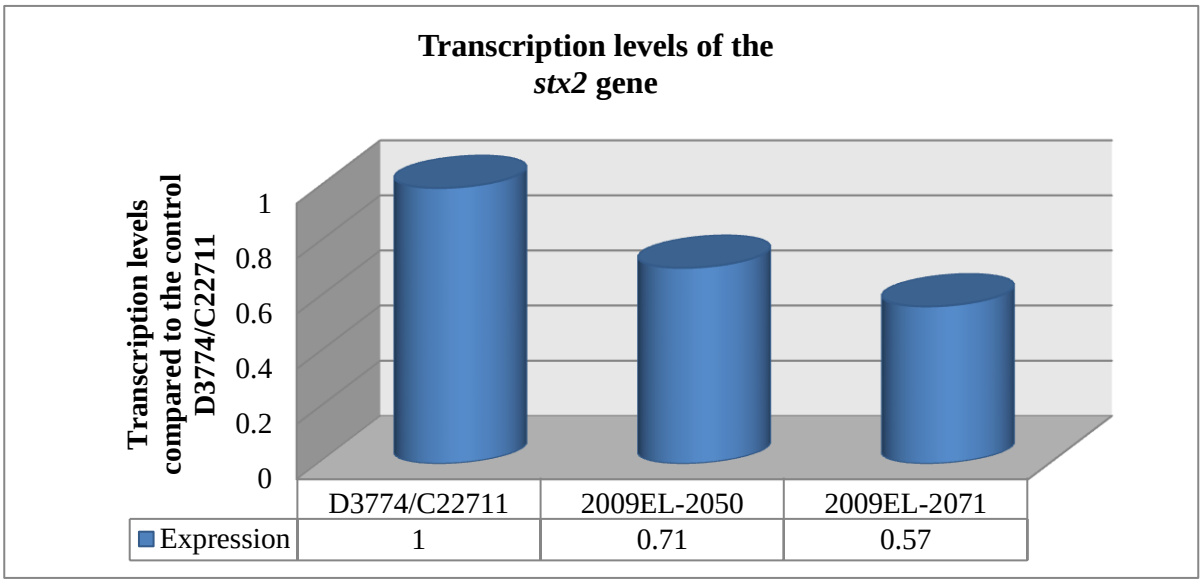


Figure 4: Transcripion levels of the *stx2* gene in the outbreak *E.coli* O104:H4 strain D3774/C22711 and the two pre-outbreak *E.coli* O104:H4 strains 2009EL -2050 and 2009EL-2071.

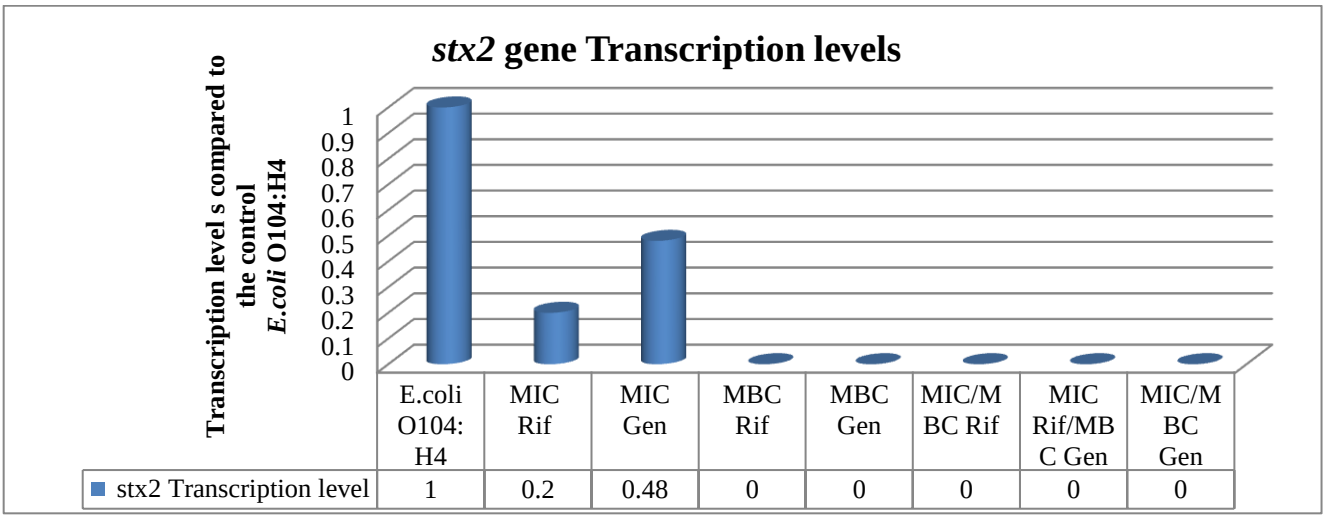


Figure 5: Transcription levels of the *stx2* gene in the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with rifampicin, gentamicin, or both at the MIC and MBC levels; Rif: rifampicin, Gen: gentamicin.

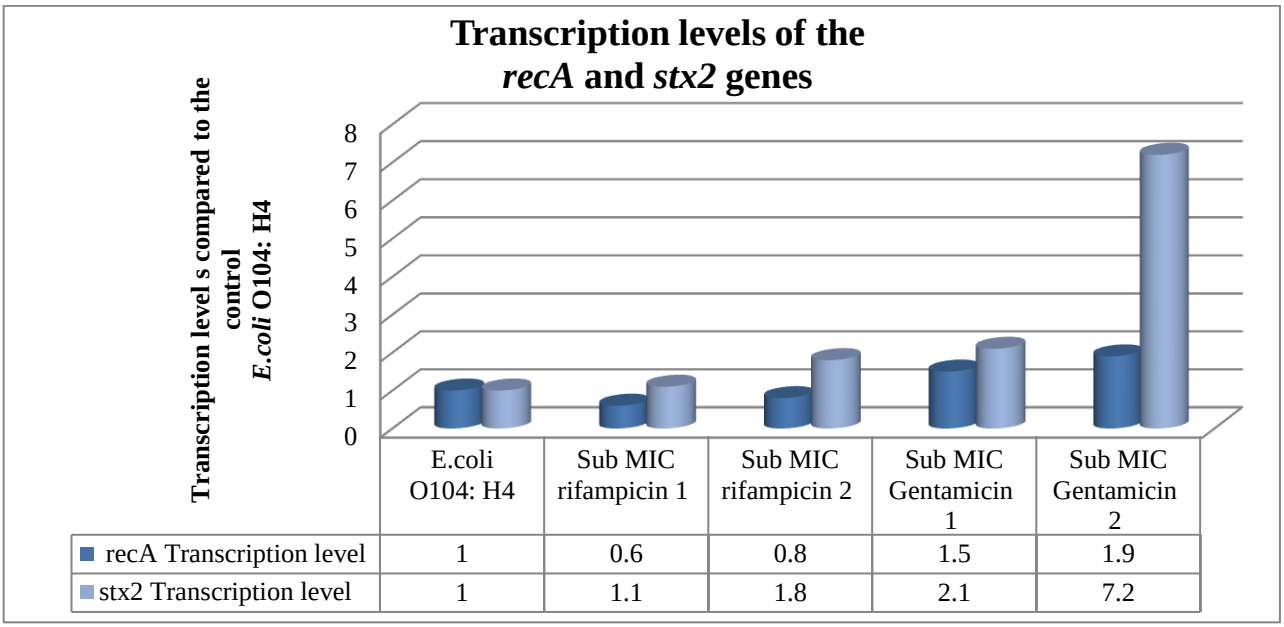


Figure 6: Transcription levels of the *recA* and *stx2* genes in the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with sub- inhibitory concentrations of rifampicin and gentamicin.

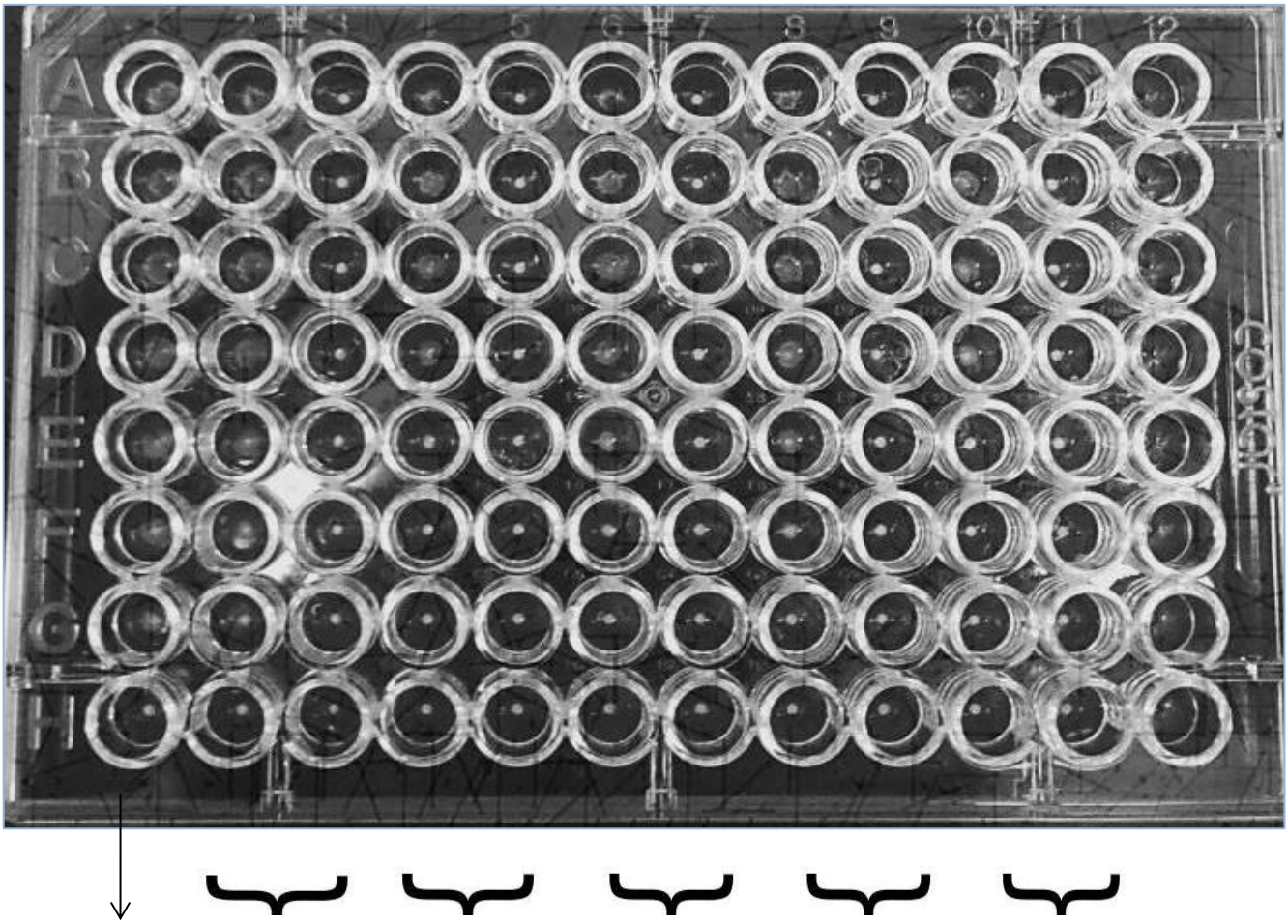


Figure 7: Reverse Passive Latex Agglutination Assay (RPLA) for Stx2 in supernatant of the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with different regimens of rifampicin and gentamicin.

Lane 1 is the positive control for Stx2. Lane 2 and 3 contain *E.coli* O104:H4 with anti-Stx2 and Latex control respectively. Lane 4 and 5 contain *E.coli* O104:H4 subjected to the MIC of rifampicin with anti- Stx2 and Latex control respectively. Lane 6 and 7 contain *E.coli* O104:H4 subjected to the MBC of rifampicin with anti- Stx2 and Latex control respectively. Lane 8 and 9 have *E.coli* O104:H4 subjected to the MIC of gentamicin with anti- Stx2 and Latex control respectively. Lane 10 and 11 contain *E.coli* O104:H4 subjceted to the MBC of Gentamicin with anti- Stx2 and Latex control respectively. Titer is 1/2 in row A, 1/4 in row B, 1/8 in row C, 1/16 in row D, 1/32 in row E, 1/64 in row F, 1/128 in row G and Negative control in row H; Rif: rifampicin, Gen: gentamicin.

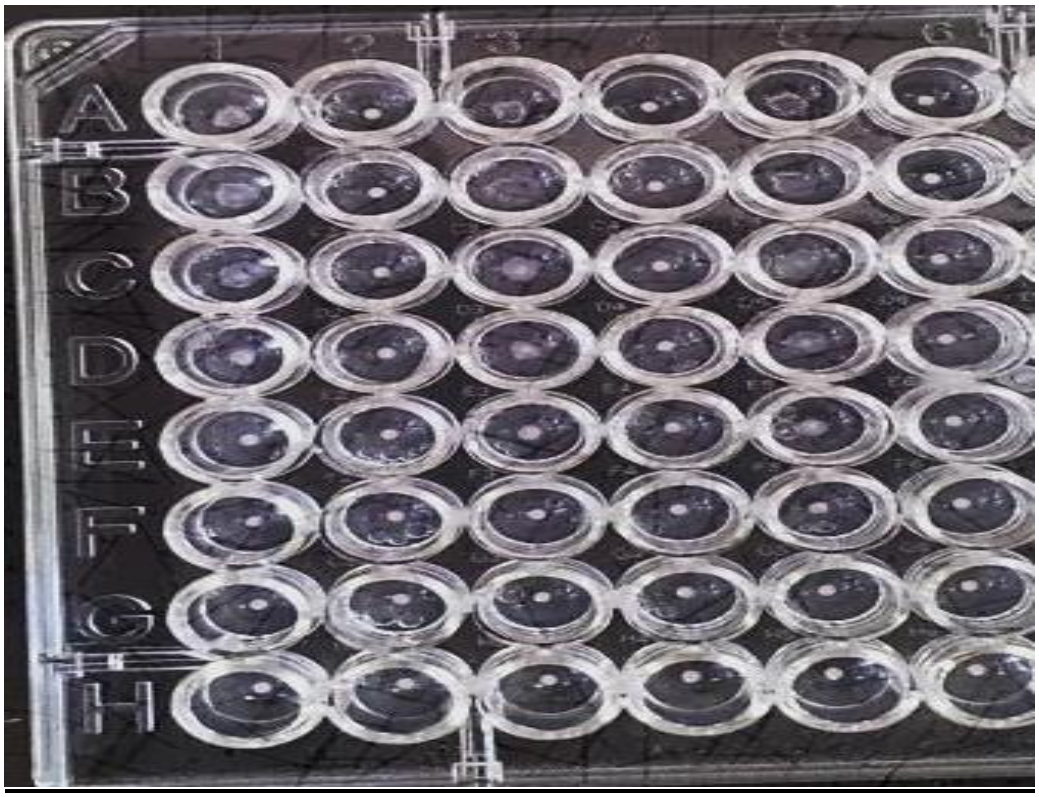


Figure 8: Reverse Passive Latex Agglutination Assay (RPLA) for Stx2 in supernatant of the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with different regimens of rifampicin and gentamicin.

Lane 1 and 2 contain *E.coli* O104:H4 subjected to the MIC of rifampicin followed by its MBC with anti- Stx2 and Latex control respectively. Lane 3 and 4 have *E.coli* O104:H4 subjected to the MIC of rifampicin followed by the MBC of gentamicin with anti- Stx2 and Latex control respectively. Lane 5 and 6 contain *E.coli* O104:H4 subjected to the MIC of gentamicin followed by its MBC with anti- Stx2 and Latex control respectively. Titer is 1/2 in row A, 1/4 in row B, 1/8 in row C, 1/16 in row D, 1/32 in row E, 1/64 in row F, 1/128 in row G and Negative control in row H; Rif: rifampicin, Gen: gentamicin.

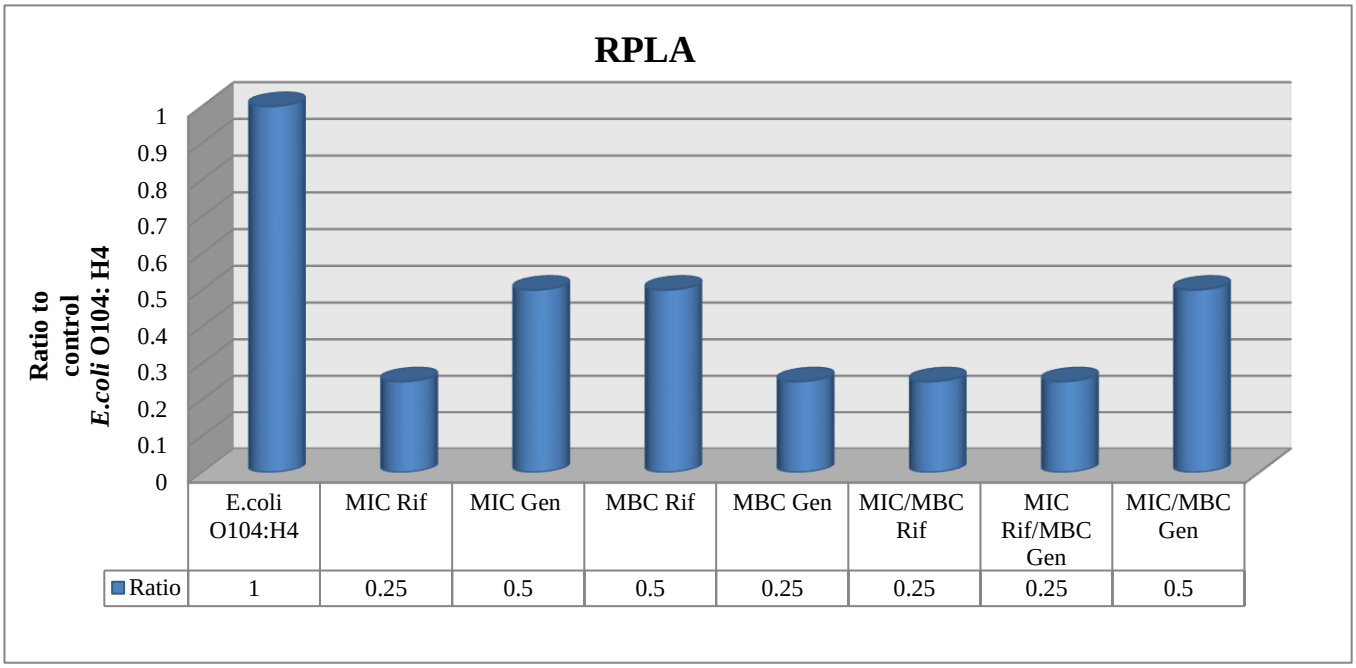


Figure 9: Reverse Passive Latex Agglutination Assay (RPLA) for Stx2 in supernatant of the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with different combinations of rifampicin and gentamicin; Rif: rifampicin, Gen: gentamicin.

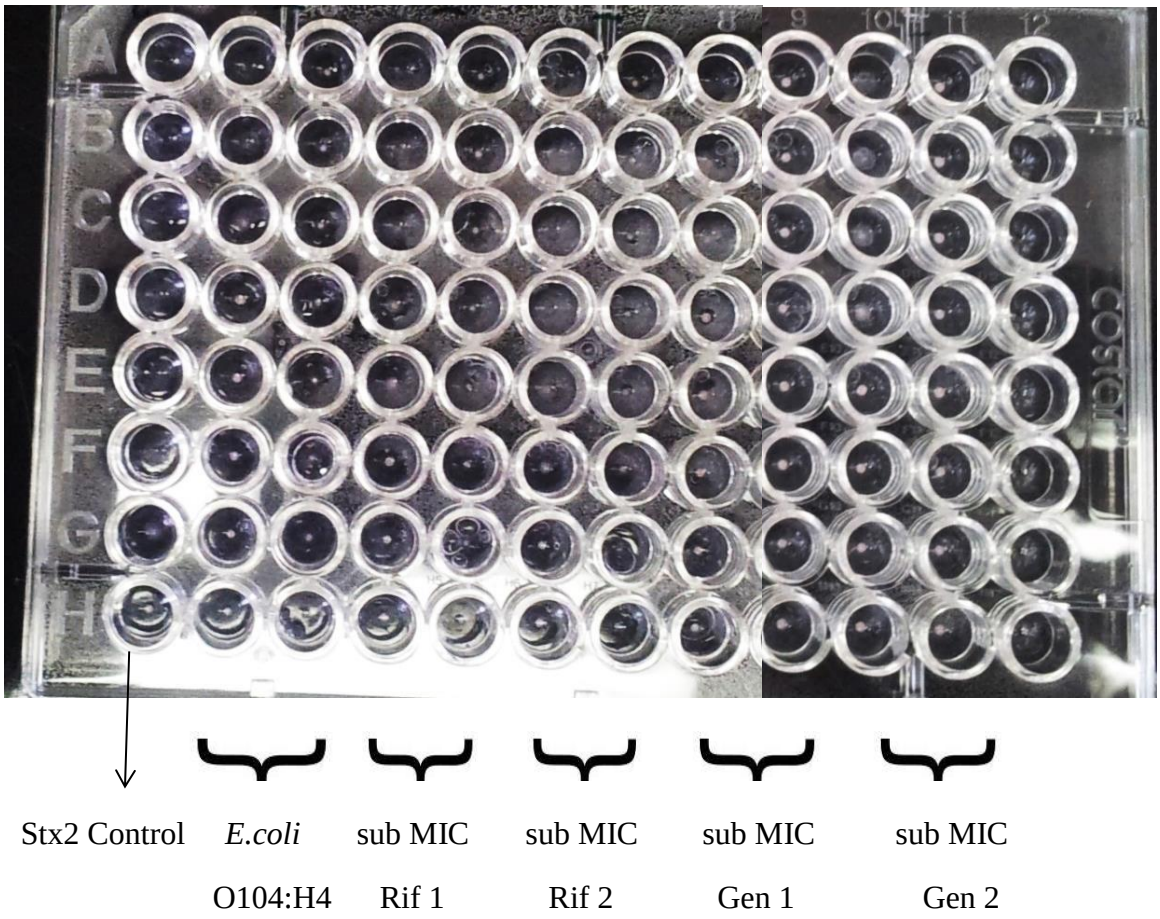


Figure 10: Reverse Passive Latex Agglutination Assay (RPLA) for Stx2 in supernatant of the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with sub-inhibitory concentrations of rifampicin and gentamicin.

Lane 1 is the positive control for Stx2. Lane 2 and 3 contain *E.coli* O104:H4 with anti-Stx2 and Latex control respectively. Lane 4 and 5 contain *E.coli* O104:H4 subjected to the sub MIC 1 of rifampicin with anti- Stx2 and Latex control respectively. Lane 6 and 7 contain *E.coli* O104:H4 subjected to the sub MIC 2 of rifampicin with anti- Stx2 and Latex control respectively. Lane 8 and 9 contain *E.coli* O104:H4 subjected to the sub MIC 1 of Gentamicin with anti- Stx2 and Latex control respectively. Lane 10 and 11 contain *E.coli* O104:H4 subjected to the sub MIC 2 of gentamicin with anti- Stx2 and Latex control respectively .Titer is 1/2 in row A, 1/4 in row B, 1/8 in row C, 1/16 in row D, 1/32 in row E, 1/64 in row F, 1/128 in row G and Negative control in row H; Rif: rifampicin, Gen: gentamicin, sub MIC: sub- inhibitory concentration.

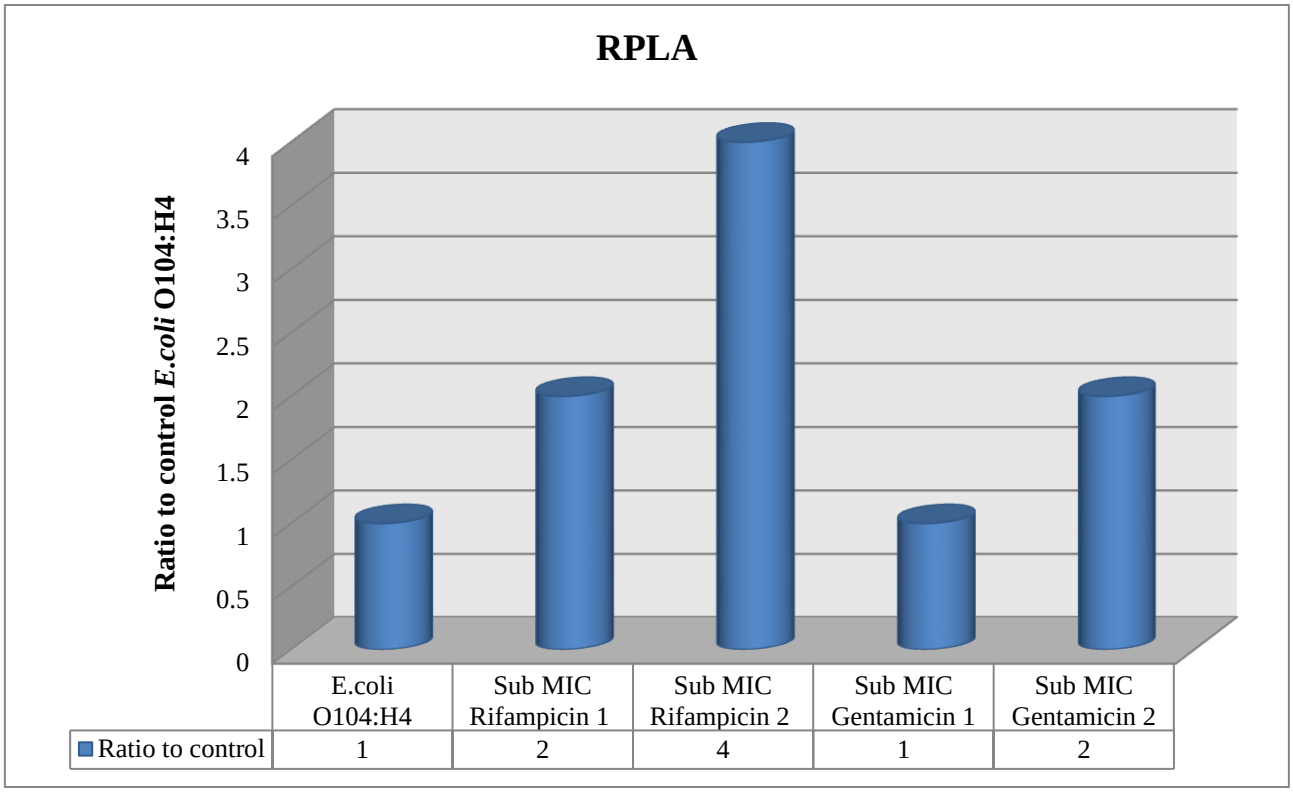
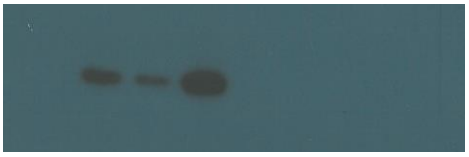


Figure 11: Reverse Passive Latex Agglutination Assay (RPLA) for Stx2 in supernatant of the outbreak *E.coli* O104:H4 strain D3774/C22711 subjected to sub-inhibitory concentrations of rifampicin and gentamicin.

1 2 3 4 5



LexA protein

L9 ribosomal protein

Figure 12: Western Blotting results for the outbreak *E.coli* O104:H4 D3774/C22711 strain treated with sub- inhibitory concentrations of rifampicin and gentamicin.

Lane 1: Control: *E.coli* O104:H4

Intensity of 1 in LexA protein: 1.0298×10^{3} pixels

Intensity of 1 in L9 ribosomal protein: 3.9896×10^{3} pixels

Ratio of Lane 1: 0.25812

Lane 2: *E.coli* O104:H4+ Sub MIC 1 of rifampicin

Intensity of 2 in LexA protein: 9.6783×10^{2} pixels

Intensity of 2 in L9 ribosomal protein: 9.8678×10^{3} pixels

Ratio of Lane 2: 0.09808

Lane 3: *E.coli* O104:H4+ Sub MIC 2 of rifampicin

Intensity of 3 in LexA protein : 6.4132×10^{3} pixels

Intensity of 3 in L9 ribosomal protein: 2.2602×10^{4} pixels

Ratio of Lane 3: 0.2837

Lane 4: *E.coli* O104:H4+ Sub MIC 1 of gentamicin

Intensity of 4 in LexA protein: 0 pixels

Intensity of 4 in L9 ribosomal protein: 1.5329×10^4 pixels

Ratio of Lane 3: 0

Lane 5: *E.coli* O104:H4+ Sub MIC 2 of gentamicin

Intensity of 5 in LexA protein: 0 pixels

Intensity of 5 in L9 ribosomal protein: 1.3664×10^4 pixels

Ratio of Lane 3: 0

➔ Ratios of Lanes 2,3,4,5 compared to the control:

E.coli O104:H4+ Sub MIC 1 of rifampicin: 0.4

E.coli O104:H4+ Sub MIC 2 of rifampicin: 1.1

E.coli O104:H4+ Sub MIC 1 of gentamicin: 0

E.coli O104:H4+ Sub MIC 2 of gentamicin: 0

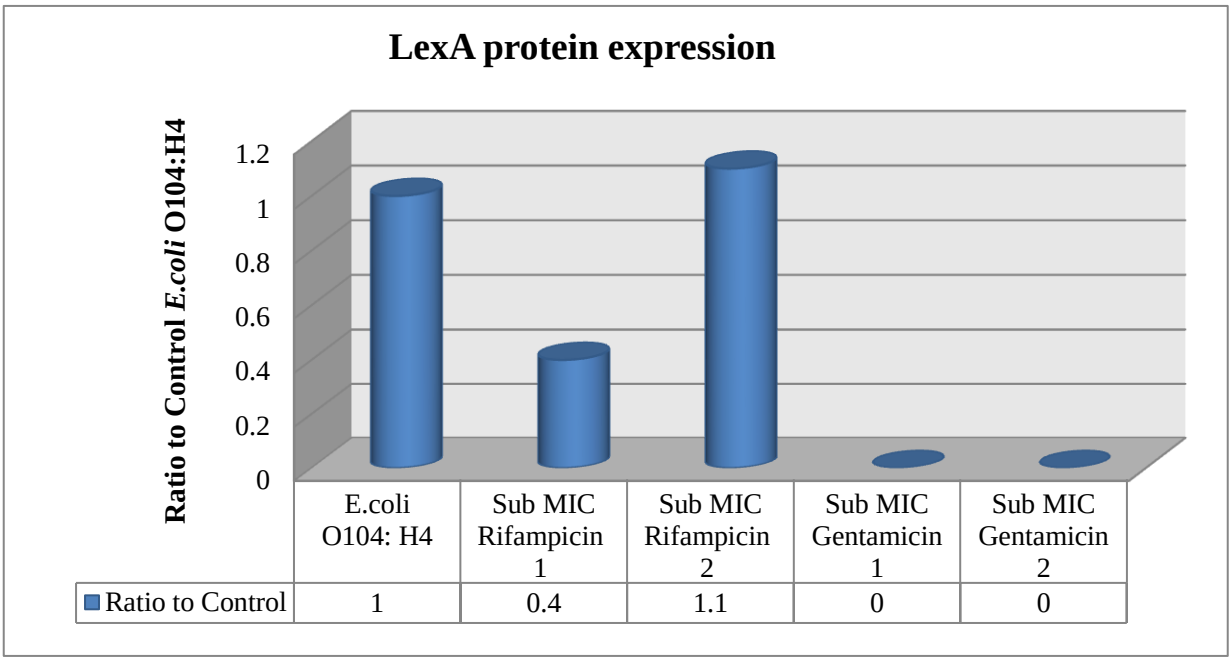


Figure 13: LexA Protein expression in the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with sub-inhibitory concentrations of rifampicin and gentamicin

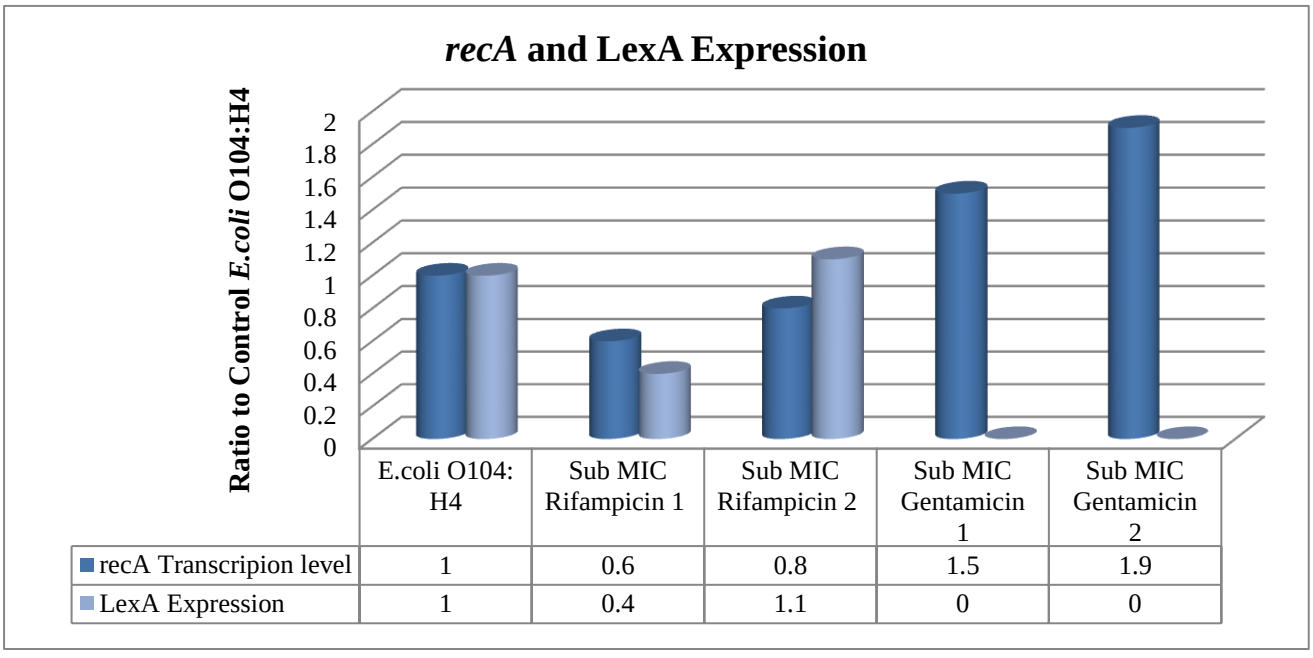
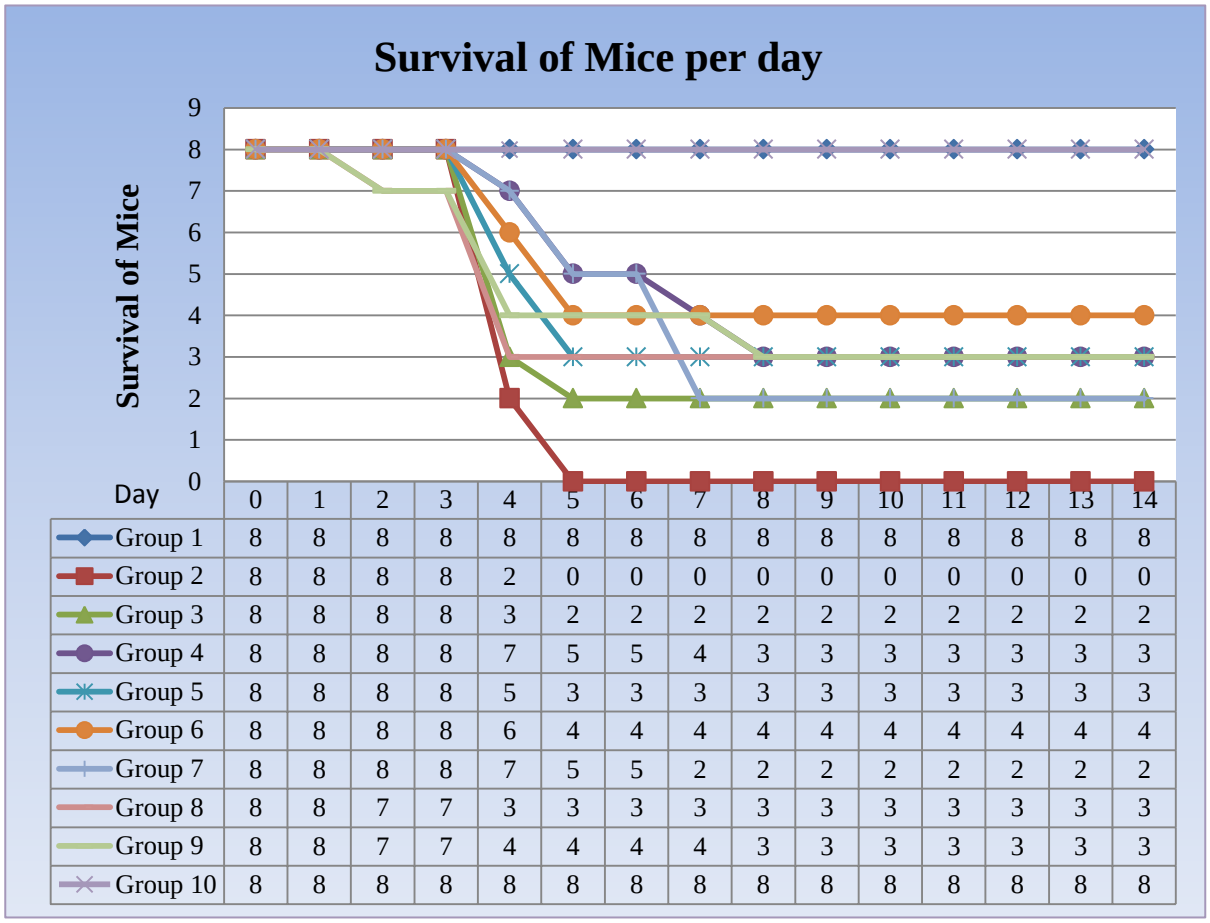


Figure 14: Expression of the LexA protein and transcription levels of the *recA* gene in the outbreak *E.coli* O104:H4 strain D3774/C22711 treated with sub-inhibitory concentrations of rifampicin and gentamicin



	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group 1	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Group 2	8	8	8	8	2	0	0	0	0	0	0	0	0	0	0
Group 3	8	8	8	8	3	2	2	2	2	2	2	2	2	2	2
Group 4	8	8	8	8	7	5	5	4	3	3	3	3	3	3	3
Group 5	8	8	8	8	5	3	3	3	3	3	3	3	3	3	3
Group 6	8	8	8	8	6	4	4	4	4	4	4	4	4	4	4
Group 7	8	8	8	8	7	5	5	2	2	2	2	2	2	2	2
Group 8	8	8	7	7	3	3	3	3	3	3	3	3	3	3	3
Group 9	8	8	7	7	4	4	4	4	3	3	3	3	3	3	3
Group 10	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8

Figure 15: BALB/c mouse survival after infection with the outbreak *E.coli* O104:H4 strain D3774/C22711 and treatment with different combinations of rifampicin and gentamicin

- ✓ Group 1: TSB/TSB/TSB
- ✓ Group 2: 3 ×LD_{50} *E.coli* O104:H4,
- ✓ Group 3: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin
- ✓ Group 4: 3 ×LD_{50} *E.coli* O104:H4/ MBC rifampicin
- ✓ Group 5: 3 ×LD_{50} *E.coli* O104:H4/ MIC gentamicin
- ✓ Group 6: 3 ×LD_{50} *E.coli* O104:H4/ MBC gentamicin
- ✓ Group 7: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin/ MBC rifampicin
- ✓ Group 8: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin/ MBC gentamicin
- ✓ Group 9: 3 ×LD_{50} *E.coli* O104:H4/ MIC gentamicin/ MBC gentamicin
- ✓ Group 10: TSB/ MIC rifampicin/ MBC gentamicin

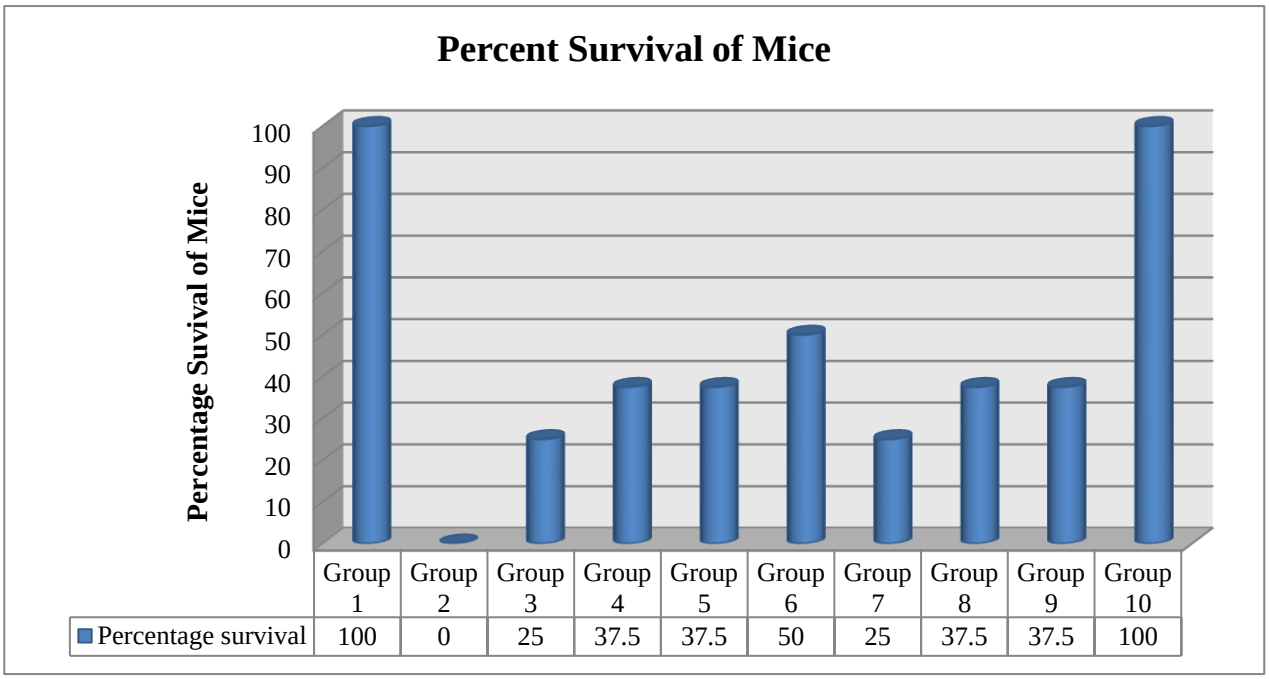


Figure 16: Percent survival of BALB/c mice infected with the outbreak *E.coli* O1O4:H4 strain D3774/C22711 and treated with different regimens of rifampicin and gentamicin; Rif: rifampicin, Gen: gentamicin.

- ✓ Group 1: TSB/TSB/TSB
- ✓ Group 2: 3 ×LD_{50} *E.coli* O104:H4,
- ✓ Group 3: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin
- ✓ Group 4: 3 ×LD_{50} *E.coli* O104:H4/ MBC rifampicin
- ✓ Group 5: 3 ×LD_{50} *E.coli* O104:H4/ MIC gentamicin
- ✓ Group 6: 3 ×LD_{50} *E.coli* O104:H4/ MBC gentamicin
- ✓ Group 7: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin/ MBC rifampicin
- ✓ Group 8: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin/ MBC gentamicin
- ✓ Group 9: 3 ×LD_{50} *E.coli* O104:H4/ MIC gentamicin/ MBC gentamicin
- ✓ Group 10: TSB/ MIC rifampicin/ MBC gentamicin

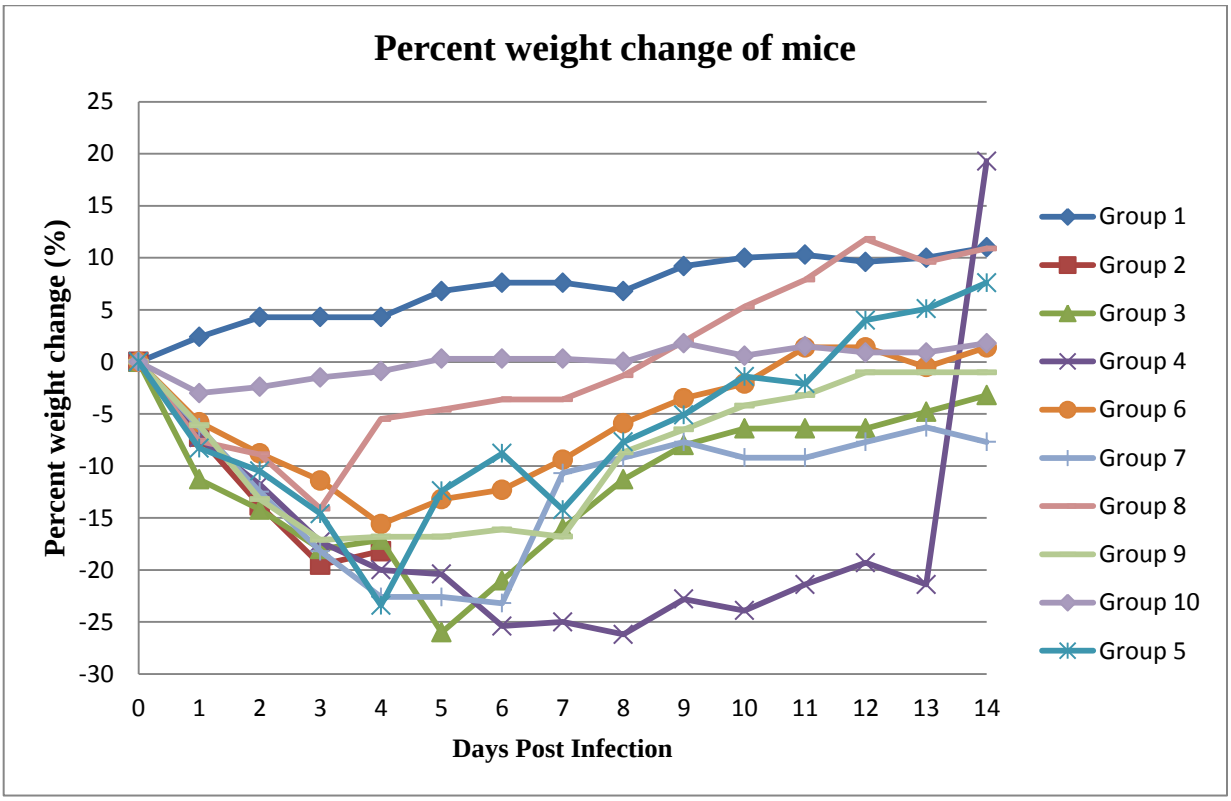


Figure 17: Percent weight change for BALB/c mice infected with the outbreak *E. coli* O104:H4 strain D3774/C22711 and treated with different combinations of rifampicin and gentamicin.

- ✓ Group 1: TSB/TSB/TSB
- ✓ Group 2: 3 ×LD_{50} *E.coli* O104:H4,
- ✓ Group 3: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin
- ✓ Group 4: 3 ×LD_{50} *E.coli* O104:H4/ MBC rifampicin
- ✓ Group 5: 3 ×LD_{50} *E.coli* O104:H4/ MIC gentamicin
- ✓ Group 6: 3 ×LD_{50} *E.coli* O104:H4/ MBC gentamicin
- ✓ Group 7: 3 ×LD_{50} *E.coli* O104:H4/ MIC rifampicin/MBC rifampicin
- ✓ Group 8: 3 ×LD_{50} *E.coli* O104:H4/MIC rifampicin/MBC gentamicin
- ✓ Group 9: 3 ×LD_{50} *E.coli* O104:H4 MIC gentamicin/MBC gentamicin
- ✓ Group 10: TSB/ MIC rifampicin/MBC gentamicin

CHAPTER V

DISCUSSION

Shiga toxin producing *E.coli* O104:H4 is a rare serogroup and only sporadic cases have been reported in humans (43). In 2009, two strains of Shiga toxin producing *E.coli* O104:H4 from cases of bloody diarrhea in the Republic of Georgia, 2009EL-2050 and 2009EL-2071, were isolated. During this study, genomic relatedness between the new pathotype causing outbreak in 2011 in Germany and the two pre-outbreak strains from the Republic of Georgia was determined using PFGE. The similarity between the outbreak and the pre-outbreak strains suggests that the outbreak strain could have been originated from the Georgian strains. In addition, the 3 different strains might have diverged recently from a common ancestor. A study performed by Ahmad et al. showed that the Georgian isolates are the closest genomically to the outbreak strain so far, however, several differences in their prophages, genomic islands, and plasmids are evident (169). Moreover, a model was proposed for the evolution of the Georgian and the outbreak strains suggesting that the 3 strains recently diverged from a common ancestor. The model also suggests that the *stx2* encoding phage that was initially present in the common ancestor was displaced by a second *stx2* encoding phage in the outbreak strain, since the genome of the *stx2* phage differ in the pre-outbreak and outbreak strains (169). Our study also showed that the transcription levels of the *stx2* gene differed between the Georgian and the outbreak strains. A study conducted by Wagner et al. showed that *E.coli* strains harboring diverse *stx2* phages resulted in a wide range of Stx2 toxin production especially in drug free cultures, demonstrating the effect of phage genotype on toxin production (170).

Antimicrobial treatment of Shiga toxin producing *E.coli* infections has been controversial, as it is associated with an increased frequency of HUS (154, 171, 172). Antimicrobial agents may augment the production of Stxs from STEC strains due to bacterial lysis and consequent release of Stxs (97). During the 2011 outbreak, the use of antimicrobial agents as a mode of treatment was not recommended. However, since the outbreak Shiga toxin producing *E.coli* O104:H4 is a hybrid pathotype of EHEC and EAEC harboring unique characteristics, potential modes of treatment using different regimens of rifampicin, an antimicrobial agent that suppresses RNA synthesis, and gentamicin, an antibiotic that affects protein synthesis, were assessed. In addition, previous studies performed in the Department of Experimental Pathology, Immunology and Microbiology, also presented evidence of a potential mode of treatment on *E.coli* O157:H7 which involved employing an antimicrobial agent that would inhibit toxin expression prior to treatment with a bactericidal antimicrobial agent. This combination was also included in the present study to determine its effectiveness (173, 174).

The effect of rifampicin, gentamicin, or a combination of both at the MIC and MBC levels on the transcript level of the *stx2* gene was determined. The *stx2* gene transcript level was completely inhibited in *E.coli* O104:H4 samples treated with the combination treatments namely the MIC of rifampicin followed by its MBC, the MIC of rifampicin followed by the MBC of gentamicin, the MIC of gentamicin followed by its MBC, and in *E.coli* O104:H4 samples subjected to either the MBC of rifampicin or the MBC of gentamicin. This indicates that these combinations and doses were effective in inhibiting the transcription of the *stx2* gene. *E.coli* O104:H4 treated with either the MIC of rifampicin or the MIC of gentamicin resulted in a decrease in the *stx2* gene transcript levels; however,

the decrease in the transcription level in gentamicin was not as high as in rifampicin. This might be due to the effect of gentamicin on growth rather than on RNA transcription. The effect of the antimicrobial agents on the toxin release was also determined. All doses and combinations showed a decrease in the release of Stx2 into the growth medium when compared to the control. This can be explained by the fact that Stx2, unlike Stx1, is not stored in the periplasmic space, rather it is released into the extracellular fraction (89). Therefore, upon addition of the MBC dose of the antimicrobial agents and rupture of the bacteria, no stored Stx2 was released.

The different treatment regimens applied *in vitro* were tested *in vivo*, in a mouse model. By day 5 post infection, none of the mice in the group that received the lethal dose of *E.coli* O104:H4 only remained alive. Groups of mice that were treated with rifampicin, gentamicin, or both seemed to benefit from antimicrobial therapy and the survival rate ranged from 25% to 50%. Groups that received either the MIC or MBC of gentamicin had a higher survival rate than groups that were treated with either the MIC or MBC of rifampicin respectively. A study conducted by Tarr et al. showed that *E.coli* O157:H7 developed resistance to rifampicin when used singly as therapy (175). The highest survival rate was observed in the group of infected mice that received the MBC of gentamicin. In contrast, the *in vivo* study done by Rahal et al. showed that all mice died in a group that was first infected with *E.coli* O157:H7 and then received the MBC dose of gentamicin. In addition, the treatment of the group of mice infected with *E.coli* O104:H4 with the MIC of rifampicin followed by the MBC of gentamicin was effective; however, the survival rate was slightly lower than that observed in the study done by Rahal et al. (173, 174). The difference in the survival rate between the two studies can be explained by the fact that

E.coli O157:H7 can produce Stx1, which is stored in the periplasmic space, and Stx2, which is released to the extracellular media. Therefore, gentamicin mediated elimination of *E.coli* O157:H7 might rupture of the bacteria and lead to enhanced release of stored toxins, making the infection worse. Gentamicin does not seem to affect the outbreak *E.coli* O104:H4 strain similarly, since the bacteria produces Stx2 only.

Few studies were conducted concerning the effect of antimicrobial agents on Shiga toxin release in the outbreak strain *E.coli* O104:H4. The effect of the several antimicrobial agents including rifampicin and gentamicin at 0.064× MIC, 0.016× MIC, 0.25× MIC, 1× MIC, and 4× MIC levels on *E.coli* O104:H4 and *E.coli* O157:H7 were compared in a study conducted by Corogeanue et al. (52). Rifampicin at the 1× MIC concentration did not enhance the transcription levels of the *stx2* gene in *E.coli* O104:H4 and *E.coli* O157:H7. Moreover, rifampicin resulted in an increased release of Stx2 in in *E.coli* O104:H4 and *E.coli* O157:H7. In comparison, our study demonstrated similar results with regard to the transcription levels of the *stx2* gene; however, the results were different regarding the Stx2 release (decrease in the Stx2 levels). In addition, the study by Corogeanue et al. demonstrated that gentamicin at the 1× MIC level led to a slightly high *stx2* gene transcript levels in *E.coli* O104:H4. However, it resulted in a decline in the *stx2* gene transcription levels in *E.coli* O157:H7. In addition, gentamicin at the MIC level did not enhance the release of Stx2 in *E.coli* O104:H4 and *E.coli* O157:H7. Moreover, the study showed that antimicrobial agents such as meropenem, chloramphenicol, fosfomycin, and ciprofloxacin can be used in the treatment of *E.coli* O104:H4, as they do not enhance the release of Stx2. In comparison, the present study showed that gentamicin at the MIC level had a diminishing effect on the *stx2* gene transcription levels and Stx2 release in *E.coli* O104:H4.

Enhanced toxin production in *E.coli* could also be due to the induction of the *stx*-encoding prophages, activated by the SOS response (97). The SOS response can be activated by a number of factors, one of which is antimicrobial agents (176). Antimicrobial agents initially administered at the MIC or supra MIC dose eventually reach sub MIC levels in tissues until the next administration (177). Subsequently, the effect of the sub MIC levels of antimicrobial agents on the induction of release of Stx2 via the SOS response was assessed.

During this study, gentamicin at both sub MIC levels induced a high *recA* gene transcript levels, completely inhibited the LexA protein expression, and led to a high *stx2* gene transcript levels, indicating that the SOS response was activated. On the other hand, the Stx2 release was doubled at the sub MIC 2 level and was equal at the sub MIC 1 level in comparison to the control. The *stx2* gene transcript levels at both sub MICs were higher when compared to the Stx2 release. This could be due to the mode of action of gentamicin, as it usually affects protein synthesis. Similarly, the study by Corogeanue et al. demonstrated that gentamicin at 0.25× MIC level led to an increased *stx2* gene transcript level in *E.coli* O104:H4. However, the Stx2 production was reduced at this sub MIC level (52). Similar results with gentamicin in one strain of *E.coli* O104:H4 were observed in another study done by Bielaszewska et al.; however, these results were insignificant (161). A study done by Nassar et al. demonstrated that gentamicin at the sub MIC levels did not activate the SOS response in *E.coli* O157:H7, however, the Stx2 release was high. This was attributed to the liberation of formed Stxs in vesicles by the disruption of the lipid bilayer (178). Although gentamicin at the MIC and MBC levels proved to be effective in treating the outbreak strain, gentamicin at the sub MIC levels might activate the SOS response and

lead to an increased release of Stx2. Therefore, gentamicin should be further assessed prior to employment in the treatment of the outbreak strain infections.

Rifampicin at the sub MIC 1 level resulted in a lower *recA* gene transcript level, decreased LexA expression and a slightly higher transcript level of the *stx2* gene when compared to the control. This indicates that the SOS was not activated. The transcription level of the *recA* gene was low (LexA and RecA are inversely related), possibly due to the lag period between the transcription of the *recA* gene and its translation. This could also be due to a possibility that an intermediate DNA damage and consequently an intermediate level of the LexA protein can trigger the expression of some DNA repair genes (such as the *urvB* gene) without affecting the expression of the *recA* gene (143). On the other hand, rifampicin at the sub MIC 2 level led to a low *recA* gene transcript level, high LexA protein production, and a high transcription level of the *stx2* gene, indicating that the SOS response was not activated. In addition, rifampicin at both MIC levels resulted in a high Stx2 release. The magnitude of the increase in the Stx2 release was higher than that in the *stx2* gene transcript level. Although rifampicin inhibits mRNA synthesis, which is expected to limit protein synthesis, there is a delay in protein inhibition. The rate of RNA degradation (transit time of RNA polymerase) and protein synthesis (lifetime of mRNA) are not necessarily correlated and the rate of the second is higher such that some mRNA remain for some time after being produced (179). Although the SOS response was not activated in rifampicin at the sub MIC levels, the *stx2* transcript levels and Stx2 release was high. This could be as a result of the existence of additional processes that induce the *stx2* phage other than the SOS response. A study done by Wegrzyn et al. showed that rifampicin has less effect on sigma^{32} dependent promoters, responsible for the heat shock response , than the

primary sigma (sigma^{70}) (180). In addition, the heat shock response can be activated by antimicrobial agents, which in turn can induce the lambda phage (such as the *stx* phage) by a mechanism that is different from the SOS response (181, 182). The study by Corogeanue et al. demonstarted that sub MIC levels of rifampicin induced high *stx2* transcript levels and similarly increased Stx2 release in *E.coli* O104:H4 (52); similar results were obtained in the present study. The study done by Nassar et al. showed that the SOS response was not activated in *E.coli* O157:H7 exposed to two sub MIC levels of rifampicin, however, the *stx2* gene transcript levels and Stx2 release were high when compared to the control.

In conclusion, the treatment of the outbreak Shiga toxin producing *E.coli* O104:H4 with antimicrobial agents seems to be beneficial. This provides a promising ground for treatment of this agent. However, further studies are required to support these results. In addition, as previous studies have indicated, the effect of antimicrobial agents is dependent on the strain, concentration of the drug and the mode of action of the antibacterial agents.

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