

Effect of Rifampicin and Gentamicin on Shiga Toxin 2 Expression Level and the SOS Response in *Escherichia coli* O104:H4

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Abstract

Background: A novel pathotype, Shiga toxin-producing *Escherichia coli* O104:H4, was the cause of a severe outbreak that affected European countries, mainly Germany, in 2011. The effect of different regimens of rifampicin and gentamicin were evaluated to determine possible treatment modes for the novel strain, and to evaluate the SOS response and its effect on toxin release.

Materials and Methods: Pulsed-field gel electrophoresis (PFGE) was performed on the novel *E. coli* O104:H4 pathotype and two pre-outbreak *E. coli* O104:H4 CDC strains. Transcript levels of the *stx2* and *recA* gene (SOS response inducer) were evaluated using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) in the novel *E. coli* O104:H4 samples subjected to different regimens of rifampicin and gentamicin. Consequently, reverse passive latex agglutination (RPLA) was used to determine the Stx2 titers in these samples. Western blot was performed to determine the LexA levels (SOS response repressor) in *E. coli* O104:H4. The efficacy of treatment with antimicrobial agents was assessed in BALB/c mice.

Results: The outbreak and pre-outbreak strains are closely related as shown by PFGE, which demonstrated slight genomic differences between the three 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 subinhibitory levels. RPLA data were in accordance with the qRT-PCR results. *E. coli* O104:H4 exposed to gentamicin at both sub-minimum inhibitory concentration (MIC) levels led to high transcription levels of the *recA* gene and lack of expression of the LexA protein, implying that the SOS response was activated. Rifampicin at both sub-MIC levels resulted in low transcript levels of the *recA* gene, indicating that the SOS response was not induced. *In vivo*, the highest survival rate in BALB/c mice was observed in the group that was treated with the minimum bactericidal concentration (MBC) of gentamicin.

Conclusion: The use of antimicrobial agents in *E. coli* O104:H4 infection seems to be effective at the MIC and MBC levels. This provides a promising ground for treatment of *E. coli* O104:H4.

Background

ESCHERICHIA COLI O104:H4 was the cause of a 2011 outbreak that affected 14 European countries, Canada, and the United States (Brzuszkiewicz *et al.*, 2011; Robert Koch Institute, 2011). Globally, 4137 cases including 50 deaths were reported, the majority (96.5%) of which were centered in Germany (World Health Organization, 2011). After much debate as to the source of this outbreak, the

pathogen was traced to a shipment of fenugreek seeds imported from Egypt in 2009 (Grad *et al.*, 2012). The outbreak was associated with bloody diarrhea, hemorrhagic colitis, and an unusually high rate of hemolytic uremic syndrome (HUS) (25%), which is a potentially fatal disease (Borgatta *et al.*, 2012). The virulence of this strain arises from its novel hybrid pathotype that exhibits enteroaggregative *E. coli* characteristics and enterohemorrhagic *E. coli*'s (EHEC) ability to produce Stx2 encoded by the *stx2* gene (Bloch

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et al., 2012). The outbreak *E. coli* O104:H4 strain does not produce Stx1; it produces Stx2 only. Prior to the outbreak, eight cases of Shiga toxin-producing *E. coli* O104:H4 have been reported globally (ECDC and EFSA, 2011; Scavia *et al.*, 2011).

Treatment of infection with Shiga toxin-producing *E. coli* (STEC), including *E. coli* O104:H4, is mainly supportive (Borgatta *et al.*, 2012). The use of antimicrobial agents in the treatment of STEC is controversial due to its association with an increased risk of HUS development. The use of antimicrobial agents in the treatment of such infections might exacerbate the disease by causing lysis of the bacterial cell, resulting in enhanced release of 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 (Kimmitt *et al.*, 2000). The SOS response system is made up of more than 50 genes; however, its inducer is the RecA protein and its repressor is the LexA protein (Boutry *et al.*, 2013).

Earlier studies conducted on *E. coli* 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 subinhibitory concentrations (sub-MICs) did not activate the SOS response in *E. coli* O157:H7, denoting that these antimicrobial agents are efficient for treatment purposes (Rahal *et al.*, 2011b; Rahal *et al.*, 2012; Nassar *et al.*, 2013). Consequently, this study aimed at determining whether antimicrobial agents can be used in the treatment of the novel *Escherichia coli* O104:H4 strain infections. Therefore, the effects of rifampicin and gentamicin were evaluated at the levels of MIC and MBC *in vitro* and *in vivo*. Antimicrobial agents initially administered at the MIC or supra-MIC dose eventually reach sub-MIC levels in tissues until the next administration (Reaze *et al.*, 2006). Therefore, the effects of rifampicin and gentamicin were evaluated at two sub-MIC levels to assess the SOS response and its effect on toxin release. In addition, the relatedness of the outbreak and two pre-outbreak strains was assessed.

Materials and Methods

Bacterial strains

The outbreak strain D3774/C22711, which was isolated during the 2011 outbreak, was obtained from Statens Serum Institut in Denmark. The two pre-outbreak strains 2009EL-2050 and 2009EL-2071 were acquired from the Centers for Disease Control and Prevention (CDC). These strains were isolated from cases with bloody diarrhea in the Republic of Georgia in 2009.

Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out using *Xba*I enzyme on the outbreak and the two pre-outbreak *E. coli* O104:H4 strains to determine their genomic relatedness using the standard operating procedure for PulseNet PFGE of *E. coli* O157:H7, *E. coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei*, and *S. flexneri* (Ribot *et al.*, 2006).

MIC and MBC

The MIC and MBC of rifampicin and gentamicin for the outbreak strain D3774/C22711 was determined using the broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2009).

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Three different protocols were followed to prepare the bacterial suspensions from which RNA was extracted.

1. First protocol: To assess the difference in the *stx2* gene transcription levels, a bacterial suspension of 1×10^6 colony-forming units (CFU)/mL of each of the outbreak and the two pre-outbreak strains were inoculated into 1-mL Mueller Hinton II Broth and the samples were incubated at 37°C for 16 h.
2. Second protocol: In order to study the effects of rifampicin, gentamicin, or both at the MIC and MBC levels, on the outbreak *E. coli* O104:H4 strain, a number of samples (samples 1–8) were prepared as shown in Table 1.
3. Third protocol: To determine the effects of subinhibitory concentrations of rifampicin and gentamicin on the outbreak *E. coli* O104:H4 strain, another set of samples was prepared (samples 9–13 in Table 1). All incubations took place in a shaker incubator at 37°C for 2 h only with vigorous agitation, since the SOS response remains activated for about an hour following its induction, after which it terminates (Janion, 2008).

RNA was extracted from the following: (1) 10^6 CFU of each of the samples in the first and second protocols; and (2) 10^8 CFU of each of the samples in protocol 3 using the Illustra RNAspin Mini RNA Isolation Kit (GE, Amersham Place, United Kingdom) according to the manufacturer's specifications for bacterial cells. The QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) was then used to synthesize cDNA following the manufacturer specifications from the extracted RNA adjusted to have a concentration of 10 µg/mL.

Successively, RT-PCR for (1) the *stx2* gene in all the samples from the three protocols and (2) the *recA* gene in the samples in protocol 3, using the QuantiFast™ SYBER® green PCR kit (Qiagen, Germany), was performed. The primers were obtained from Thermo Scientific (Ulm, Germany), and were described in previous studies (Jinneman *et al.*, 2003; Shilpakala *et al.*, 2009). Reaction mixtures of 20 µL for each sample were prepared and each sample was run in triplicates for the gene in inquiry (*stx2* or *recA*) and the housekeeping gene (*rpoB*). The housekeeping gene was *rpoB*; this has been used in several studies to assess the transcription levels of *stx2* and resulted in reliable data (Rahal *et al.*, 2011b; Chen *et al.*, 2013; Nassar *et al.*, 2013). Each reaction mixture contained the following: $\times 1$ QuantiFast SYBR Green PCR master mix, 5 µM of the forward and reverse *stx2* primers, and 1100-ng/µL cDNA samples. Incubation conditions of a single cycle of 95°C for 15 min, 45 cycles of: 95°C for 10 s, Ta for 30 s, and 72°C for 20 s were achieved using BioRad CFX96 Real Time System, C1000 Thermal Cycler (Munich, Germany). The transcription level of the gene in question was calculated using the BioRad CFX manager software in either

TABLE 1. *ESCHERICHIA COLI* O104:H4 OUTBREAK STRAIN D3774/C22711 SAMPLES SUBJECTED TO DIFFERENT ANTIMICROBIAL TREATMENT PREPARED FOR REAL-TIME POLYMERASE CHAIN REACTION

Samples	
1	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 grown in Mueller Hinton II broth for 18 h
2	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 incubated with the MIC dose of rifampicin for 18 h
3	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 incubated with the MIC dose of gentamicin for 18 h
4	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 grown with the MBC dose of rifampicin for 18 h
5	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 incubated with the MBC dose of gentamicin for 18 h
6	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 grown with the MIC dose of rifampicin for 18 h, cells were centrifuged at 5000 rpm for 5 min, supernatant was discarded and the pellet was resuspended with the MBC dose of rifampicin and incubated for 4 more h
7	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 incubated with the MIC dose of rifampicin for 18 h, cells were centrifuged at 5000 rpm for 5 min, supernatant was discarded, and the pellet was resuspended with the MBC dose of gentamicin and incubated for 4 more h
8	10 ⁶ CFU/mL <i>E. coli</i> O104:H4 grown with MIC dose of gentamicin for 18 h, cells were centrifuged at 5000 rpm for 5 min, supernatant was discarded and the pellet was resuspended with MBC dose of gentamicin and incubated for 4 more h
9	2.5 × 10 ⁷ CFU/mL <i>E. coli</i> O104:H4 grown alone
10	2.5 × 10 ⁷ CFU/mL <i>E. coli</i> O104:H4 incubated with the sub-MIC 1 dose of rifampicin (2-fold dilution of the MIC dose)
11	2.5 × 10 ⁷ CFU/mL <i>E. coli</i> O104:H4 incubated with the sub-MIC 2 dose of rifampicin (4-fold dilution of the MIC dose)
12	2.5 × 10 ⁷ CFU/mL <i>E. coli</i> O104:H4 incubated with the sub-MIC 1 dose of gentamicin (2-fold dilution of the MIC dose)
13	2.5 × 10 ⁷ CFU/mL <i>E. coli</i> O104:H4 incubated with the sub-MIC 2 dose of gentamicin (4-fold dilution of the MIC dose)

CFU, colony-forming units; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

the outbreak strain sample compared with the pre-outbreak strain samples in the first protocol or samples treated with antimicrobial agents compared to samples without antimicrobial agent in the second and third protocols, employing the *rpoB* gene as a standard.

Reverse passive latex agglutination (RPLA)

RPLA was carried out on two different sets of samples. The first set included the outbreak *E. coli* O104:H4 strain exposed to rifampicin, gentamicin, or broth at the MIC and MBC levels as shown in Table 2 (samples 1–8). The growth inhibition in the samples treated with the MIC or MBC doses

of the antimicrobial agents was taken into consideration by making sure that the bacterial density of the drug-free sample was equal to that of the samples treated with the antimicrobial agents. The second set comprised the outbreak *E. coli* O104:H4 strain subjected to two sub-MICs of rifampicin and gentamicin, as shown in Table 2 (samples 9–13). RPLA was performed using VTEC-RPLA kit (Oxoid, Tokyo, Japan) in order to compare the differential production of Stx2 in these samples.

BALB/c mice

Adult, male and female, 4–8-week-old mice weighing 19–41 g were obtained from the Animal Care Facility at the

TABLE 2. *ESCHERICHIA COLI* O104:H4 OUTBREAK STRAIN D3774/C22711 SAMPLES SUBJECTED TO DIFFERENT REGIMENS OF THE ANTIMICROBIAL AGENTS PREPARED FOR REVERSE PASSIVE LATEX AGGLUTINATION

Samples	
Sample 1:	The outbreak <i>E. coli</i> O104:H4 strain grown in antimicrobial-free TSB for 20 h at 37°C
Sample 2:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the MIC dose of rifampicin for 20 h at 37°C
Sample 3:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the MBC dose of rifampicin for 20 h at 37°C
Sample 4:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the MIC dose of gentamicin for 20 h at 37°C
Sample 5:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the MBC dose of gentamicin for 20 h at 37°C
Sample 6:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the MIC dose of rifampicin for 16 h at 37°C, followed by the addition of the MBC dose of rifampicin for 4 h
Sample 7:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the MIC dose of rifampicin for 16 h at 37°C, followed by the addition of the MBC dose of gentamicin for 4 h
Sample 8:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the MIC dose of gentamicin for 16 h at 37°C, followed by the addition of the MBC dose of gentamicin for 4 h
Sample 9:	The outbreak <i>E. coli</i> O104:H4 strain grown in antimicrobial free TSB for 20 h at 37°C
Sample 10:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the sub-MIC 1 dose of rifampicin for 20 h at 37°C
Sample 11:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the sub-MIC 2 dose of rifampicin for 20 h at 37°C
Sample 12:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the sub-MIC 1 dose of gentamicin for 20 h at 37°C
Sample 13:	The outbreak <i>E. coli</i> O104:H4 strain grown in TSB containing the sub-MIC 2 dose of gentamicin for 20 h at 37°C

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; TSB, trypticase soy broth.

TABLE 3. MICE GROUPS AND TREATMENT REGIMENS ON THE OUTBREAK *ESCHERICHIA COLI* O104:H4 STRAIN D3774/C22711

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 <i>E. coli</i> O104:H4	—	—
Group 3	3 × LD ₅₀ of <i>E. coli</i> O104:H4	MIC rifampicin	—
Group 4	3 × LD ₅₀ of <i>E. coli</i> O104:H4	MBC rifampicin	—
Group 5	3 × LD ₅₀ of <i>E. coli</i> O104:H4	MIC gentamicin	—
Group 6	3 × LD ₅₀ of <i>E. coli</i> O104:H4	MBC gentamicin	—
Group 7	3 × LD ₅₀ of <i>E. coli</i> O104:H4	MIC rifampicin	MBC rifampicin
Group 8	3 × LD ₅₀ of <i>E. coli</i> O104:H4	MIC rifampicin	MBC gentamicin
Group 9	3 × LD ₅₀ of <i>E. coli</i> O104:H4	MIC gentamicin	MBC gentamicin
Group 10 (drug control)	TSB	MIC rifampicin	MBC gentamicin

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; TSB, trypticase soy broth.

American University of Beirut. The mice were cared for and handled according to “Guide for the Care and Use of Laboratory Animals” (National Research Council Committee, 2011). Although the preferable route of mouse inoculation is oral, intraperitoneal infection was used since BALB/c mice are resistant to STEC infection via the oral route as was done in previous studies (Rahal *et al.*, 2011a, 2011b, 2012). STEC do not colonize the mouse gastric system unless oral antimicrobial agents are given. The use of additional antimicrobials would bias the results since the purpose of the study is to assess the effects of particular antimicrobial agents by themselves in a controlled experimental setting. The LD₅₀ of *E. coli* O104:H4 in BALB/c mice was identified using the method described by Nowotny (Nowotny, 1979). Subsequently, 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 was assessed. As a result, 80 mice were divided into 10 groups, as shown in Table 3. The *in vivo* MIC and MBC equivalent doses were determined as described in the previous studies. A control group mock treated with sterile broth and a drug control group that received sterile free broth and antimicrobial agents were included. 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 first injections were administered at the beginning of the experiment (hour 0), the second injections were given 1 h later (hour 1), and the third injections were administered 16 h after the second injections (hour 17). The mice were monitored for a period of 2 weeks for weight loss and survival.

Western blotting

Protein assay. Protein was extracted from samples of the outbreak strain grown in antimicrobial-free broth or subjected to sub-MIC 1 and 2 of rifampicin and gentamicin using bacterial lysis buffer prepared as previously described

(Nassar *et al.*, 2013). Bradford assay was then carried out to determine the concentration of the extracted proteins.

Western blot. Western blotting was done to study the expression of the LexA protein and L9 ribosomal protein (loading/ housekeeping protein control) levels using previously described antibodies (Nassar *et al.*, 2013).

Statistical analysis

Statistical analysis was done for the transcription levels of the *stx2* and *recA* genes using unpaired *t*-test using the Graph-Pad *t*-test calculator. In addition, the Kaplan–Meier method was used to estimate the survival rates for each group and the log rank (Mantel–Cox) test was used to compare them. All *p*-values < 0.05 were considered to be statistically significant.

Results

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 (Fig. 1). The pulsetype profiles obtained using the outbreak and pre-outbreak Georgian strains 2009EL-2071 and 2009EL-2050 are similar to those reported by Scheutz *et al.* (Scheutz *et al.*, 2011). However, the codes provided in Scheutz *et al.* are different from those provided by the Statens Serum Institut in Denmark for the outbreak strain and the CDC for the pre-outbreak strains.

qRT-PCR

The qRT-PCR analysis indicated that the *stx2* gene was expressed in the outbreak and pre-outbreak strains. In addition, the *stx2* gene transcript level in the outbreak strain was 1.41 times (*p*-value: 0.0001) and 1.75 times (*p*-value: 0.0001) that of the 2009 EL-2050 and 2009 EL-2071 pre-



FIG. 1. Dendrogram of the pulsed-field gel electrophoresis gel for the outbreak *Escherichia coli* O104:H4 strain D3774/C22711 and the two pre-outbreak *E. coli* O104:H4 strains 2009EL-2050 and 2009EL-2071.

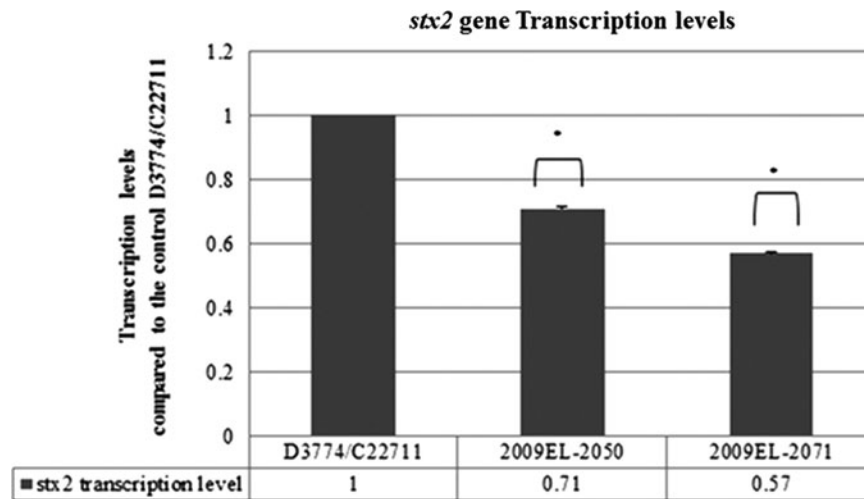


FIG. 2. Transcription levels of the *stx2* gene in the outbreak *Escherichia coli* O104:H4 strain D3774/C22711 and the two pre-outbreak *E. coli* O104:H4 strains 2009EL-2050 and 2009EL-2071. Standard error of the mean values: D3774/C22711: 0.0110, 2009EL-2050: 0.00396, and 2009EL-2071: 0.00576. **p*-value < 0.05.

outbreak strains, respectively (Fig. 2). Furthermore, qRT-PCR analysis showed that the treatment of the outbreak strain with the MIC of rifampicin (16 $\mu\text{g}/\text{mL}$) caused an 80% decrease in the transcript levels of the *stx2* gene in comparison to the control (*E. coli* O104:H4 without antimicrobial agent) (*p*-value: 0.0448). Conversely, complete inhibition of the transcription of the *stx2* gene was observed upon treatment of the outbreak strain with the following: the MBC of rifampicin (32 $\mu\text{g}/\text{mL}$) (*p*-value: 0.009), the MBC of gentamicin (4 $\mu\text{g}/\text{mL}$) (*p*-value: 0.0230), 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 decrease in the *stx2* transcript level was observed in the sample of the outbreak *E. coli* O104:H4 treated with the MIC of gentamicin (1 $\mu\text{g}/\text{mL}$) (*p*-value: 0.0128) (Fig. 3).

qRT-PCR results showed that the outbreak *E. coli* O104:H4 samples subjected to the sub-MIC 1 and 2 of rifampicin (8 $\mu\text{g}/$

mL and 4 $\mu\text{g}/\text{mL}$, respectively) led to a lower *recA* (*p*-value: 0.0369 and 0.3881, respectively) and higher *stx2* transcript levels (*p*-value: 0.8993 and 0.5674, respectively) in comparison to the control. On the other hand, gentamicin at the sub-MIC 1 and 2 concentrations (0.5 $\mu\text{g}/\text{mL}$ and 0.25 $\mu\text{g}/\text{mL}$ respectively), resulted in a 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 (Fig. 4).

RPLA

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 twofold or fourfold decrease in Stx2 release in comparison with the control, as presented in Figure 5. On the other hand, treating the outbreak strain with the sub-MIC 1 and 2 of rifampicin resulted in an increase in

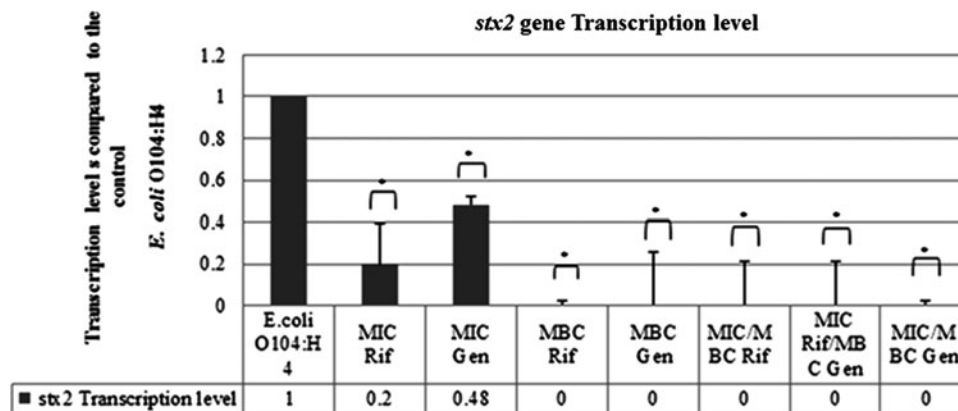


FIG. 3. Transcription levels of the *stx2* gene in the outbreak *Escherichia coli* O104:H4 strain D3774/C22711 treated with rifampicin, gentamicin, or both at the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) levels. SEM values: *E. coli* O104:H4: 0.11430, MIC Rif: 0.19482, MIC Gen: 0.03904, MBC Rif: 0.0212, MBC Gen: 0.25431, MIC/MBC Rif: 0.20874, MIC Rif/MBC Gen: 0.20989, MIC/MBC Gen: 0.0212. Rif, rifampicin; Gen, gentamicin. **p*-value < 0.05.

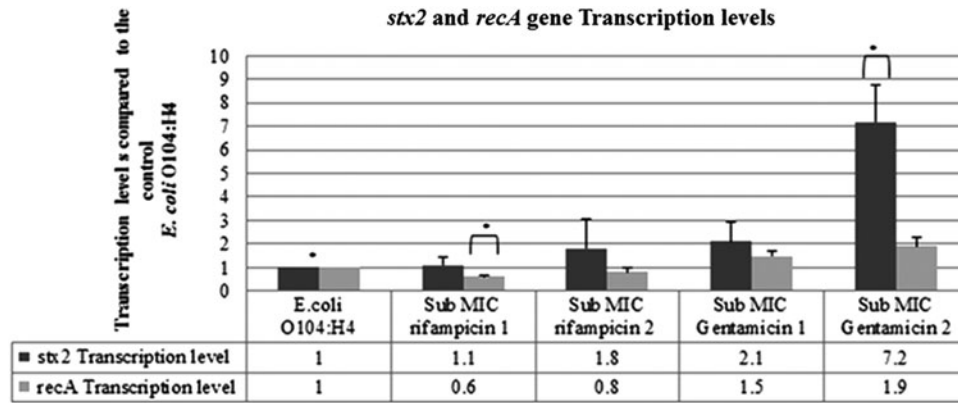


FIG. 4. Transcription levels of the *recA* and *stx2* genes in the outbreak *Escherichia coli* O104:H4 strain D3774/C22711 treated with subinhibitory concentrations of rifampicin and gentamicin. SEM: *E. coli* O104:H4: *stx2* gene: 0.16209, *recA* gene: 0.11461, sub-minimum inhibitory concentration (MIC) rifampicin 1: *stx2* gene: 0.33558, *recA* gene: 0.08335, sub-MIC rifampicin 2: *stx2* gene: 1.28469, *recA* gene: 0.17978, sub-MIC gentamicin 1: *stx2* gene: 0.0859, *recA* gene: 0.20212, and sub-MIC gentamicin 2: *stx2* gene: 0.0744, *recA* gene: 0.36856. **p*-value < 0.05.

Stx2 release when compared to the control. Gentamicin at the sub-MIC 1 level produced an equal release of Stx2 in comparison to the control, whereas gentamicin at the sub-MIC 2 level resulted in a twofold increase in the release of Stx2 when compared to the control (Fig. 5).

Western blotting

The results of a Western blot assessing the relative levels of LexA in the outbreak strain exposed to the sub-MIC levels of the antimicrobial agents indicated that the expression of this protein was completely inhibited at both sub-MICs of gentamicin (Fig. 6). In addition, rifampicin at the sub-MIC 1 level led to a low expression of the LexA protein when compared to the nonantimicrobial-agent-treated control. However, the sub-MIC 2 of rifampicin resulted in a higher expression of the LexA protein than the control.

Mice treatment

The LD₅₀ of the outbreak *E. coli* O104:H4 strain was determined and was found to be 5.16×10^6 CFU. The $3 \times$ LD₅₀ dose was then used for assessing the effect of antimicrobial

treatment in infected mice. All mice that did not receive the bacterial injections (those in the control groups) survived the monitoring 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 (Fig. 7). During the monitoring period, the highest survival percentage (50%) was observed in the group of infected mice that received the MBC of gentamicin. A 37.5% survival rate was observed 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. The lowest survival rate (25%) 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 (Fig. 7). The results were statistically significant according to the log rank (Mantel-Cox) test (*p*-value: 0.001).

Discussion

STEC O104:H4, is a rare serotype and only sporadic cases have been reported in humans (Scheutz *et al.*, 2011). The similarity between the new pathotype and the two pre-outbreak

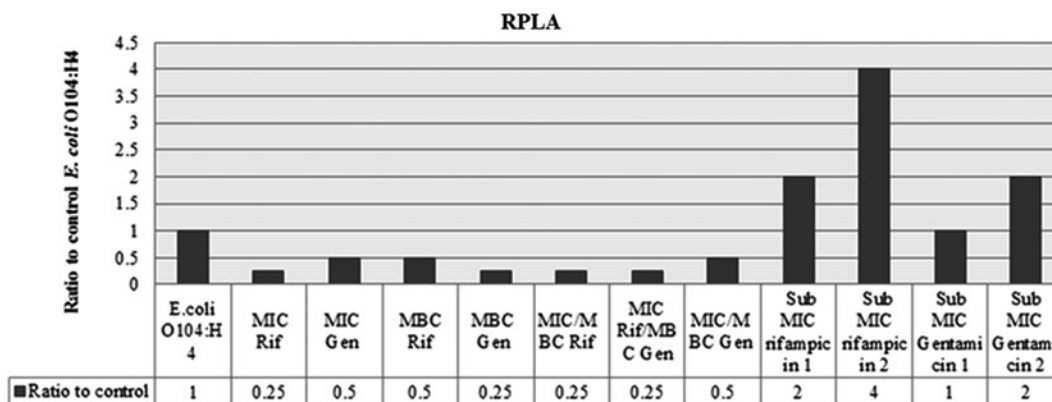


FIG. 5. Reverse passive latex agglutination assay (RPLA) for Stx2 in supernatant of the outbreak *Escherichia coli* O104:H4 strain D3774/C22711 treated with different combinations and concentrations of rifampicin and gentamicin. Rif, rifampicin; Gen, gentamicin; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

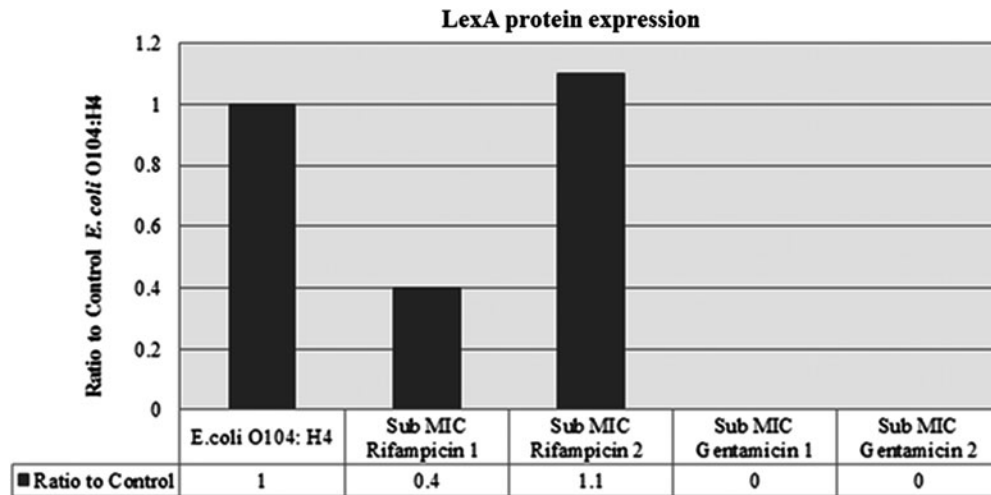


FIG. 6. LexA protein expression in the outbreak *Escherichia coli* O104:H4 strain D3774/C22711 treated with subinhibitory concentrations of rifampicin and gentamicin. MIC, minimum inhibitory concentration.

strains determined by PFGE in this study suggests that the three different strains might have diverged recently from a common ancestor. Our results are in accordance with those of the study performed by Ahmad *et al.*, which showed that the Georgian isolates are the closest genomically to the outbreak strain so far; however, several differences in their *stx2* prophages, genomic islands, and plasmids are evident (Ahmed *et al.*, 2012). Furthermore, our study showed that the transcription levels of the *stx2* gene differed between the Georgian and the outbreak strains. This might be due to differences in phage genotype in the outbreak and pre-outbreak strains.

Drug-free cultures of *E. coli* strains, harboring diverse *stx2* prophages, may yield a wide range of Stx2 toxin production (Wagner *et al.*, 1999).

Antimicrobial treatment of STEC infections has been controversial, as it is associated with an increased frequency of HUS (Wong *et al.*, 2000; Dundas *et al.*, 2001; MacConachie and Todd, 2004). Antimicrobial agents may augment the production of Stxs from STEC strains due to bacterial lysis and consequent release of Stxs (Kimmit *et al.*, 2000). Potential modes of treatment using different regimens of rifampicin and gentamicin were assessed in this study. The *in vitro* results concerning the transcription levels of the *stx2* gene and Stx2 toxin production indicated that subjecting the outbreak strain to the different regimens of the antimicrobial agents at the MIC and MBC levels was effective in lowering the expression of the gene and protein. Similarly, the study done by Rahal *et al.* showed a lowering effect of different combinations of rifampicin and gentamicin at the MIC and MBC levels on the *stx2* transcription levels and Stx2 release in *E. coli* O157:H7 (Rahal *et al.*, 2011b).

The different treatment regimens applied *in vitro* were tested *in vivo*, in a mouse model. 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, possibly due to the observation that *E. coli* develops resistance to rifampicin when used singly as therapy (Tarr *et al.*, 1990). 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 mice that were initially infected with *E. coli* O157:H7 and then treated with the MBC dose of gentamicin died (Rahal *et al.*, 2011b; Rahal *et al.*, 2012). The difference between the treatment of *E. coli* O157:H7 and *E. coli* O104:H4 may be due to 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 (Shimizu *et al.*, 2009). 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

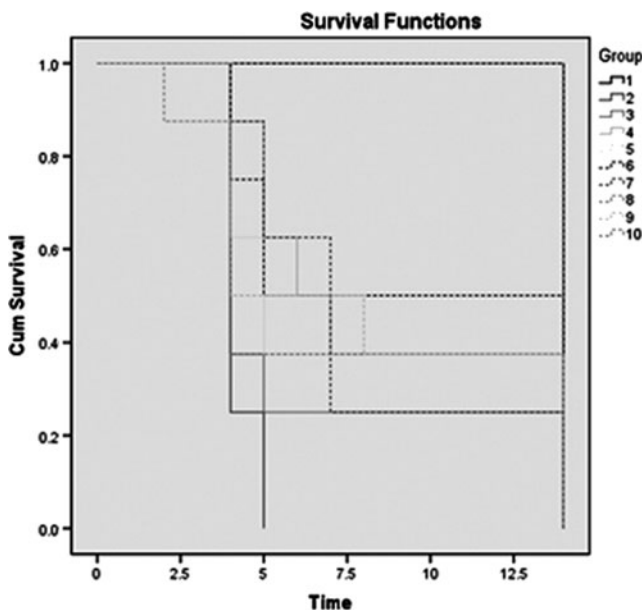


FIG. 7. Kaplan-Meier mouse survival curves after infection with the outbreak *Escherichia coli* O104:H4 strain D3774/C22711 and treatment with different combinations of rifampicin and gentamicin for the 14-day monitoring period. (Group numbers correspond to those in Table 3). Cum, cumulative.

seem to affect the outbreak *E. coli* O104:H4 strain similarly, since the bacteria produce Stx2 only.

Enhanced toxin production in *E. coli* could also be due to the induction of the *stx*-encoding prophages, activated by the SOS response (Kimmitt *et al.*, 2000). The SOS response can be activated by a number of factors, one of which is antimicrobial agents. Subsequently, the effect of the sub-MIC levels of antimicrobial agents on the induction of release of Stx2 via the SOS response was assessed. Gentamicin at both sub-MIC levels induced high *recA* gene transcript levels, completely inhibited the LexA protein expression, and led to high *stx2* gene transcript levels and Stx2 toxin release, indicating that the SOS response was activated. 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. In contrast to our study, the study by Nassar *et al.* demonstrated that gentamicin at the sub-MIC levels did not activate the SOS response in *E. coli* O157:H7 (Nassar *et al.*, 2013). Although gentamicin at the MIC and MBC levels proved to be effective in treating the outbreak strain, gentamicin at the sub-MIC levels might induce 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 both sub-MIC levels resulted in a lower *recA* gene transcript level, higher transcript level of the *stx2* gene, and toxin production when compared to the control, indicating that the SOS was not activated. In addition, the expression of the LexA protein was reduced at the sub-MIC 1 of rifampicin; however, its expression was increased at the sub-MIC 2 of the antimicrobial agent. The transcription level of the *recA* gene at the sub-MIC 1 of rifampicin was low 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 subsequently an intermediate level of the LexA protein can elicit the expression of some DNA repair genes without altering the expression of the *recA* gene (Brent, 1982). In addition, 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 (Reid and Speyer, 1970). Furthermore, the transcription of the *stx2* gene and the Stx2 release was high even though the SOS response was not activated, possibly due to the existence of additional processes that induce the *stx2* phage other than the SOS response. Sigma³²-dependent promoters, responsible for the heat shock response, are not affected by rifampicin as much as the primary sigma (sigma⁷⁰) (Wegrzyn *et al.*, 1998). The heat shock response can be activated by antimicrobial agents, which in sequence can induce the *stx* phage by a mechanism that is different from the SOS response (Foster, 2005; Rokney *et al.*, 2008). The study by Nassar *et al.* showed similar results with our study (Nassar *et al.*, 2013).

Few studies were conducted concerning the effect of antimicrobial agents including rifampicin and gentamicin at the MIC and sub-MIC levels on the *stx2* gene expression and

Stx2 release in the outbreak strain *E. coli* O104:H4. A study conducted by Corogeanu *et al.* demonstrated results similar to our study regarding the effect of rifampicin at the MIC level on the transcription levels of the *stx2* gene; however, the results were different regarding the Stx2 release (decrease in the Stx2 levels). Moreover, the study showed a similar effect of rifampicin at the sub-MIC level on the *stx2* transcription levels and Stx2 release. In addition, it indicated similar results when compared to our study when *E. coli* O104:H4 was subjected to the MIC and the sub-MIC of gentamicin (Corogeanu *et al.*, 2012). Similar results with gentamicin at the sub-MIC level in one strain of *E. coli* O104:H4 were observed in another study done by Bielaszewska *et al.*; however, these results were insignificant (Bielaszewska *et al.*, 2012).

In conclusion, the use of antimicrobial agents in *E. coli* O104:H4 infections seems to be effective and provides a promising ground for the treatment of human infections with this agent at the MIC and MBC levels. However, the use of the antimicrobial agents at the sub-MIC levels might lead to an increase in the expression of the *stx2* gene and hence not be as effective. Therefore, the effect of antimicrobial agents is dependent on the strain, concentration of the drug, and the mode of action.

Disclosure Statement

No competing financial interests exist.

References

- Ahmed SA, Awosika J, Baldwin C, Bishop-Lilly KA, Biswas B, Broomall S, *et al.* Genomic comparison of *Escherichia coli* O104:H4 isolates from 2009 and 2011 reveals plasmid, and prophage heterogeneity, including Shiga toxin encoding phage stx2. *PLoS ONE* 2012;7:e48228.
- Bielaszewska M, Idelevich EA, Zhang W, Bauwens A, Schaumburg F, Mellmann A, *et al.* Effects of antibiotics on Shiga toxin 2 production and bacteriophage induction by epidemic *Escherichia coli* O104:H4 strain. *Antimicrob Agents Chemother* 2012;56:3277–3282.
- Bloch SK, Felczykowska A, Nejman-Falenczyk B. *Escherichia coli* O104:H4 outbreak—Have we learnt a lesson from it? *Acta Biochim Pol* 2012;59:483–488.
- Borgatta B, Kmet-Lunacek N, Rello J. *E. coli* O104:H4 outbreak and haemolytic-uraemic syndrome. *Med Intensiva* 2012;36:576–583.
- Boutry C, Delplace B, Clippe A, Fontaine L, Hols P. SOS response activation and competence development are antagonistic mechanisms in *Streptococcus thermophilus*. *J Bacteriol* 2013;195:696–707.
- Brent R. Regulation and autoregulation by *lexA* protein. *Biochimie* 1982;64:565–569.
- Brzuszkiewicz E, Thurmer A, Schuldes J, Leimbach A, Liesegang H, Meyer FD, *et al.* Genome sequence analyses of two isolates from the recent *Escherichia coli* outbreak in Germany reveal the emergence of a new pathotype: Enteroggregative-haemorrhagic *Escherichia coli* (EAHEC). *Arch Microbiol* 2011;193:883–891.
- Chen ML, Hao Z, Tian Y, Zhang QY, Gao PJ, Jin JL. Different effects of six antibiotics and ten Traditional Chinese Medicines on Shiga toxin expression by *Escherichia coli* O157:H7. *Evid Based Complement Altern Med* 2013;2013: 121407.

- [CLSI] Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility for Bacteria that Grow Aerobically; Approved Standard*, 8th ed. CLSI Document M07–A8. Wayne, PA: CLSI, 2009.
- Corogeanu D, Willmes R, Wolke M, Plum G, Utermohlen O, Kronke M. Therapeutic concentrations of antibiotics inhibit Shiga toxin release from enterohemorrhagic *E. coli* O104:H4 from the 2011 German outbreak. *BMC Microbiol* 2012; 12:160.
- Dundas S, Todd WT, Stewart AI, Murdoch PS, Chaudhuri AK, Hutchinson SJ. The central Scotland *Escherichia coli* O157:H7 outbreak: Risk factors for the hemolytic uremic syndrome and death among hospitalized patients. *Clin Infect Dis* 2001;33:923–931.
- [ECDC and EFSA] European Centre for Disease Prevention and Control and European Food Safety Authority. Shiga toxin/verotoxin-producing *Escherichia coli* in humans, food and animals in the EU/EEA, with special reference to the German outbreak strain STEC O104. Stockholm: ECDC, 2011.
- Foster PL. Stress responses and genetic variation in bacteria. *Mutat Res* 2005;569:3–11.
- Grad YH, Lipsitch M, Feldgarden M, Arachchi HM, Cerqueira GC, Fitzgerald M, *et al.* Genomic epidemiology of the *Escherichia coli* O104:H4 outbreaks in Europe, 2011. *Proc Natl Acad Sci U S A* 2012;109:3065–3070.
- Janion C. Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. *Int J Biol Sci* 2008;4:338–344.
- Jinneman KC, Yoshitomi KJ, Weagant SD. Multiplex real-time PCR method to identify Shiga toxin genes stx1 and stx2 and *Escherichia coli* O157:H7/H- serotype. *Appl Environ Microbiol* 2003;69:6327–6333.
- Kimmitt PT, Harwood CR, Barer MR. Toxin gene expression by Shiga toxin-producing *Escherichia coli*: The role of antibiotics and the bacterial SOS response. *Emerg Infect Dis* 2000;6:458–465.
- MacConnachie AA, Todd WT. Potential therapeutic agents for the prevention and treatment of haemolytic uraemic syndrome in Shiga toxin producing *Escherichia coli* infection. *Curr Opin Infect Dis* 2004;17:479–482.
- Nassar FJ, Rahal EA, Sabra A, Matar GM. Effects of sub-inhibitory concentrations of antimicrobial agents on *Escherichia coli* O157:H7 Shiga toxin release and role of the SOS response. *Foodborne Pathog Dis* 2013;10:805–812.
- National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Washington DC: National Academies Press, 2011.
- Nowotny A. Determination of toxicity. In: *Basic Exercises in Immunochemistry*. New York: Springer-Verlag, Inc., 1979, pp. 303–305.
- Rahal EA, Kazzi N, Kanbar A, Abdelnoor AM, Matar GM. Role of rifampicin in limiting *Escherichia coli* O157:H7 Shiga-like toxin expression and enhancement of survival of infected BALB/c mice. *Int J Antimicrob Agents* 2011a;37:135–139.
- Rahal EA, Kazzi N, Nassar FJ, Matar GM. *Escherichia coli* O157:H7—Clinical aspects and novel treatment approaches. *Front Cell Infect Microbiol* 2012;2:138.
- Rahal EA, Kazzi N, Sabra A, Abdelnoor AM, Matar GM. Decrease in Shiga toxin expression using a minimal inhibitory concentration of rifampicin followed by bactericidal gentamicin treatment enhances survival of *Escherichia coli* O157:H7-infected BALB/c mice. *Ann Clin Microbiol Antimicrob* 2011b;10:34.
- Reaze A, Khonsari AM, Pirayeh SN. Effect of sub-inhibitory concentrations of gentamicin on the β -lactamase production of uropathogenic *Escherichia coli*. *Res J Med Med Sci* 2006; 1:63–67.
- Reid P, Speyer J. Rifampicin inhibition of ribonucleic acid and protein synthesis in normal and ethylenediaminetetraacetic acid-treated *Escherichia coli*. *J Bacteriol* 1970;104:376–389.
- Ribot EM, Fair MA, Gautom R, *et al.* Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, *Shigella* and *Shigella* for PULSENET. *Foodborne Pathog Dis* 2006;3:59–67.
- Robert Koch Institute. Report: Final presentation and evaluation of epidemiological findings in the EHEC O104:H4 outbreak, Germany 2011. Berlin 2011. Available at: http://www.rki.de/EN/Home/EHEC_final_report.pdf?__blob=publicationFile, accessed May 14, 2013.
- Rokney A, Kobiler O, Amir A, Court DL, Stavans J, Adhya S, *et al.* Host responses influence on the induction of lambda prophage. *Mol Microbiol* 2008;68:29–36.
- Scavia G, Morabito S, Tozzoli R, Michelacci V, Marziano ML, Minelli F, *et al.* Similarity of Shiga toxin-producing *Escherichia coli* O104:H4 strains from Italy and Germany. *Emerg Infect Dis* 2011;17:1957–1958.
- Scheutz F, Nielsen EM, Frimodt-Moller J, Boisen N, Morabito S, Tozzoli R, *et al.* Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. *Euro Surveill* 2011;16:19889.
- Shilpakala SR, Raghunathan M. Impact of DNA gyrase inhibition by antisense ribozymes on rec A in *E. coli*. *Mol Biol Rep* 2009;36:1937–1942.
- Shimizu T, Ohta Y, Noda M. Shiga toxin 2 is specifically released from bacterial cells by two different mechanisms. *Infect Immun* 2009;77:2813–2823.
- Tarr PI, Neill MA, Clausen CR, Watkins SL, Christie DL, Hickman RO. *Escherichia coli* O157:H7 and the hemolytic uremic syndrome: Importance of early cultures in establishing the etiology. *J Infect Dis* 1990;162:553–556.
- Wagner PL, Acheson DW, Waldor MK. Isogenic lysogens of diverse Shiga toxin 2-encoding bacteriophages produce markedly different amounts of Shiga toxin. *Infect Immun* 1999; 67:6710–6714.
- Wegrzyn A, Szalewska-Palasz A, Blaszczyk A, Liberek K, Wegrzyn G. Differential inhibition of transcription from sigma70- and sigma32-dependent promoters by rifampicin. *FEBS Lett* 1998;440:172–174.
- Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *N Engl J Med* 2000;342:1930–1936.
- World Health Organization. Outbreaks of *E. coli* O104:H4 infection: update 28. Published online July 1, 2011. Available at: <http://www.euro.who.int/en/what-we-do/health-topics/emergencies/international-health-regulations/news/news/2011/07/outbreaks-of-e.-coli-o104h4-infection-update-28>, accessed May 14, 2013.

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