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Research Article

FITNESS AND PATHOGENICITY OF OUTBREAK CAUSING *Salmonella* UPON SHORT-TERM EXPOSURE TO GROUNDWATER WITH RESIDUAL ANTIBIOTICS

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Abstract: The changes in survival and pathogenicity of three *Salmonella enterica* subsp. *enterica* serotypes upon short term exposure to groundwater with residual antibiotics have been studied in relationship to overall microbial fitness. A wild type flagellated *Salmonella enterica* ser. Typhimurium outbreak strain, a mutant *Salmonella enterica* ser. Typhimurium strain, and a wild type avian disease-causing *Salmonella enterica* ser. Pullorum strain were exposed to a range of ionic strength (3-30 mM) groundwater with residual antibiotics for 6-24 hours. Exposed organisms' pathogenicity was tested in vitro exposure to a human epithelial cell line (HEp2). Resistance profiles against 10 common antibiotics were also tested and compared to unexposed controls. Results show minor antibiotic resistance changes for *S. enterica* ser. Typhimurium strains in response to some antibiotic classes mediated with active efflux pumps. This trend was not observed for *S. enterica* ser. Pullorum, suggesting that resistance found in groundwater exposed organisms might be strain-dependent. In vitro epithelial cell invasion assays showed bacterial invasion of HEp2 cells initially decreases with time and increases after 24 hours. It is concluded that *S. enterica* serotypes reaching groundwater environments in the presence of residual antibiotics may exhibit increased levels of pathogenicity, strain-dependent resistance to antibiotics, and sustained levels of viability.

Key words: *Salmonella*, antibiotic resistance, invasion, groundwater, pathogenicity.

Özet: Bu çalışmada kalıntı miktarda antibiyotik içeren yeraltı sularına kısa süreli maruz bırakılan üç *Salmonella enterica* subsp. *enterica* serotipinin hastalık yapıcı özelliklerindeki değişiklikler ve hayatta kalabilme seviyeleri genel mikrobiyel fitnes çerçevesinde incelenmiştir. Serotipler gerçek bir salgından izole edilen kamçılı doğal fenotip *Salmonella enterica* ser. Typhimurium, kamçısı fonksiyonel olmayan bir başka *Salmonella enterica* ser. Typhimurium suşu ve kuş türlerinde salgına yol açan doğal fenotip *Salmonella enterica* ser. Pullorum'dan oluşup, iyon şiddeti değişken (3-30 mM) ve kalıntı miktarda antibiyotik içeren yeraltı suyuna 6-24 saat sürecince maruz bırakılmıştır. Serotiplerin hastalık yapıcı özellikleri in vitro koşullarda HEp2 türü insan epitel hücre kültürleri kullanılarak ölçülmüştür. Antibiyotik direnç seviyeleri ise yaygın kullanılan 10 antibitiyotiğe karşı disk difüzyon testi yardımıyla belirlenmiştir. Deneyler sonucunda *S. enterica* ser. Typhimurium suşlarında antibiyotik direnç seviyelerinde az miktarda artış gözlenirken *S. enterica* ser. Pullorum suşunda değişiklik gözlenmemiştir. In vitro deneylerde ise suşların HEp2 hücrelerini enfekte etmeleri zamanla önce azalmış, 24 saatlik dilim sonunda ise artmıştır. Sonuç olarak, kalıtsal antibiyotik içeren yeraltı sularına temas eden *S. enterica* serotiplerinin hastalık yapıcı karakterlerinin arttığı, antibiyotik direnç seviyelerinin suşa bağlı olarak değişkenlik gösterirken hayatta kalma oranlarının yüksek olduğu gözlenmiştir.

Introduction

Salmonella is a robust zoonotic pathogen threatening both human and animal health significantly. Non-typhoidal *Salmonella* infections are increasing worldwide with at least nine to ten incidents reported each year since 2009 only in the U.S. (Vugia *et al.* 2004). A recent major outbreak caused by *S. enterica* ser. Typhimurium has affected 714 individuals in 46 states (CDC 2009). One of the reservoirs for pathogenic bacteria including *Salmonella* following excretion from infected individuals is groundwater environments (Schmoll *et al.* 2006). Pathogens released from various point and non-point sources of human and/or animal waste will likely reach groundwater and create health related issues in individuals who are directly or indirectly exposed to the groundwater (Crane & Moore 1984). Considering the facts that antibiotics are frequently detected in various groundwater systems (Barber *et al.* 2009, Barnes *et al.* 2008, Vulliet & Cren-Olivé 2011) and 26% of the total freshwater demand is supplied through groundwater (Hutson 2004), it is essential to understand the effects of groundwater environments with residual antibiotics on the fitness of waterborne pathogens as it pertains to several physiological

characteristics such as pathogenicity, survivability and gained or lost resistance to certain antibiotics.

Another unknown issue with the ion-rich nature of groundwater systems is how bacterial efflux pumps are affected with respect to antimicrobial resistance, especially against aminoglyglycosides (Bradford 2008), tetracyclines (Chopra & Roberts 2001) and quinolones (Poole 2000). It has been previously documented that working mechanisms of these bacterial efflux pumps are directly and/or indirectly linked to the ionic components in the external environment. For example, the insertion of aminoglycosides into bacterial LPS (Bradford 2008) follows a proton gradient (energy) dependent transport across the cytoplasmic membrane. Additionally, a tetracycline efflux pump system is induced under the presence of Mg^{2+} in the external environment (Roberts 1996, Orth *et al.* 2000), which results in the excretion of the antibiotic in an energy-dependent manner with proton exchange for a tetracycline-cation complex (Yamaguchi *et al.* 1993). The majority of efflux pumps work in this manner, *i.e.*, compound-ion antiporter, and are reviewed in detail elsewhere (Kumar & Schweizer 2005). Similarly, efflux pump systems in *Salmonella* also assist the bacteria in resistance to antibiotics such as ciprofloxacin (Giraud *et al.* 2000), β-lactams (Nikaido *et al.* 1998), as well as creating simultaneous resistance to multiple drugs (Abouzeed *et al.* 2008, Piddock *et al.* 2000, Quinn 2006). Resistance to β-lactams in *Salmonella* is mediated by the AcrAB efflux system and AcrB is also required for virulence of *S. enterica* ser. Typhimurium (Lacroix 1996), suggesting that drug transporters may contribute not only to antibiotic resistance but also to virulence (Kunihiko Nishino 2006). Despite these findings, comprehensive studies on changes of antibiotic resistance in waterborne *Salmonella* spp. exposed to groundwater with residual antibiotics have not been performed so far.

This study aims to investigate whether the fitness of *Salmonella* is affected when exposed to ion-rich groundwater conditions with residual antibiotics in the external environment. The fitness was evaluated with respect to decreased antibiotic resistance, viability and pathogenicity against human cell lines. For these purposes, an outbreak-causing wild type *S. enterica* ser. Typhimurium, a non-motile mutant phenotype of *S. enterica* ser. Typhimurium and *S. enterica* ser. Pullorum, an important avian outbreak-causing strain, were selected as model organisms. These particular strains were chosen due to their different genotypic and phenotypic variation and their low-level susceptibility to the tested antibiotics as control groups to monitor any suspected changes in their profiles.

Materials and Methods

Bacterial strain selection and preparation

All *Salmonella* strains used in the study were obtained from the Salmonella Genetic Stock Centre (SGSC) of University of Calgary, Alberta, Canada. *S. enterica* ser. Typhimurium strain ST5383 is a wild type strain and was originally isolated from an outbreak infecting more than 1700 people (Bezanson *et al.* 1985). The strain designated as SGSC1512 is a non-motile mutant of *S. enterica* ser. Typhimurium TM2 (Yamaguchi *et al.* 1986). The non-motile, non-flagellated avian pathogenic *S. pullorum* wild type strain SA1685 (CDC number 2863-65) was originally isolated from an infected turkey.

S. enterica serotypes used during the course of the study was pre-cultured in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) at 37°C overnight, and shaken continuously at 120 rpm. Overnight grown cultures were inoculated into fresh LB broth in 1:100 dilutions and harvested at the mid-exponential phase determined by the growth curve analysis for each bacterium. A refrigerated bench-top centrifuge (5804R; Eppendorf, Hamburg, Germany) equipped with a fixed angle rotor (F-34-6-38; Eppendorf) was used to pellet the cells with an applied 3,700×*g* force for 15 min at 4°C. Growth medium was decanted and the pellet was resuspended in 10 mM potassium chloride (KCl) solution. The process was repeated twice in order to ensure complete removal of the growth medium. Electrolyte solutions used in cell preparation and other experiments were prepared with de-ionized water (DIW) (Millipore, Billerica, MA) and reagent-grade KCl (Fisher Scientific). The concentration of the cell stock solution was determined by using a cell counting hemocytometer (Bürker-Turk, Germany) under a light microscope (Fisher Scientific).

Groundwater exposure conditions

Bacteria cultured and harvested as described above were exposed to groundwater (GW) for 6, 12, 18 and 24 hours. GW was prepared with a slight modification of a previously used procedure (Bolster *et al.* 1999) by dissolving $CaCl_2.2H_2O$ (36 mg), $CaSO_4.2H_2O$ (25 mg), KNO_3 (20 mg), $NaHCO_3$ (36 mg), $Ca(NO_3)_2.4H_2O$ (35 mg) and $MgSO_4.7H_2O$ (60 mg) in 1 L of sterile deionized water (DIW) and the ionic strength (IS) of GW were adjusted to 3.33 mM (original recipe), 10 mM and 30 mM accordingly. For the test group, 1 μg/L of each the following antibiotics were added to GW: amoxicillin (MP Biomedicals), tetracycline (EMD Chemicals, Darmstadt, Germany), ampicillin (EMD Chemicals), chloramphenicol (EMD Chemicals), kanamycin (EMD Chemicals), gentamicin (MP Biomedicals), streptomycin (MP Biomedicals), sulfamethoxazole (MP Biomedicals), and penicillin (Fisher Scientific). The pH of GW was kept constant at 7.0±0.2. Bacteria suspensions of 10^8 cells per mL were exposed to the aforementioned GW conditions in 100 mL tissue culture flasks with 0.2 μm vented caps (Corning) wrapped in aluminum foil, placed on orbital shakers, and mildly shaken (70 rpm) for the tested time period. Bacterial isolates exposed to GW without the residual antibiotics were referred as the control group.

Cell viability analysis

At the end of exposure to GW, viability of both the control and the test groups was determined by using the Live/Dead BacLight® kit (L-7012; Molecular Probes,

Eugene, OR) according to the manufacturer's directions. Direct counting of the stained live and dead cells was done using an inverted microscope (IX70; Olympus, Japan) operated under red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT).

Antibiotic susceptibility analysis

Antibiotic susceptibility tests were employed to assay any change in susceptibility of *Salmonella* isolates against 10 different antimicrobial chemicals commonly used by the U.S. National Antimicrobial Resistance Monitoring System (NARMS). Tests were performed in compliance with the Clinical Laboratory Standards (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS), (CLSI 2003) for the following antimicrobial agents (numbers in parentheses denote the amount of antimicrobial chemical impregnated in 6 mm disks): amikacin (30 µg), amoxicillin-clavulanic acid (30 µg) cefoxitin (30 µg), ceftriaxone (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), tetracycline (30 µg) and trimethoprim-sulfamethoxazole (25 µg).

Epithelial cell culture and invasion assays

The human epithelial cell line HEp-2 was obtained from American Type Culture Collection (ATCC) (CCL-23; ATCC, Manassas, VA) and was grown in Eagle's Minimum Essential Medium (EMEM) (ATCC) supplemented with 10% fetal bovine serum (ATCC) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Epithelial cells were incubated at 37˚C under 5% CO_2.

Invasion of *Salmonella* into epithelial cells was quantified with slight modifications of the protocols described in Burnham *et al.* (2007). Briefly, a monolayer of HEp-2 cells was grown until confluence in 24-well plates (Corning, Corning, NY). *Salmonella,* at a concentration of 10^5 cells per mL, was inoculated into cell-culture plates and incubated for 2 hours at 37˚C to allow internalization. Following the incubation, each well of the plates was washed three times with phosphate buffered saline (PBS) to remove unbound bacteria. Bacteria that were bound to epithelial cells but had not been internalized were killed by applying fresh growth medium containing 5 µg penicillin per mL and 100 µg gentamicin per mL, and incubated for 2 hours at 37˚C. Following the incubation, cells were washed with PBS, treated with trypsin-EDTA complex (ATCC), and lysed with 0.1 % Triton-X100 (Fisher Scientific). The lysates were spread onto LB agar plates and incubated for 18 hours at 37˚C. The number of colony forming units (CFUs) was counted to quantify the number of *Salmonella* that had successfully invaded the monolayer of the epithelial cells.

Statistical analysis of data

Changes in the diameters of antibiotic inhibition zones (for determining antibiotic susceptibility profiles), viability, and the number of CFUs of *Salmonella* infecting HEp2 cells were statistically compared using the unpaired Student's t-test using Minitab® Version 14 (State College, PA). Differences between the control and the test groups were considered to be significant at 95% confidence interval ($P < 0.05$).

Light microscopy imaging of epithelial cells

Representative images of both uninfected and *Salmonella*-invaded HEp2 cells were obtained by using a light microscope (Micromaster, Fisher Scientific) (Fig. 1). The Giemsa stain (Fisher Scientific) which differentially stains human and bacterial cells in purple and pink, respectively was used. Chambered cover glass slides (Nunc Lab-Tek, Fisher Scientific) were used to grow HEp2 cells until desired confluence and infected with *Salmonella* as described above. After the chambered cover glasses were manually removed, the slides were placed in Giemsa stain (2.5%) for 45-60 minutes. Slides were rinsed by dipping in a buffer solution [0.59% (w/v) Na_2HPO_4, 0.36% (w/v) $NaH_2PO_4H_2O$] three-four times and left to air dry. Stained slides were observed under light microscope and their images were taken at 400× magnification.

Results

Antibiotic susceptibility analysis

The control group of the non-motile mutant SGSC1512 was susceptible to all antibiotics except cephalothin and nalidixic acid, where intermediate level resistance was observed (Table 1). SGSC1512 exposed to GW for 6, 12, 18 and 24 hours did not change in resistance to antibiotics except for cephalothin to which the isolates became susceptible after exposure periods of 12, 18, and 24 hours at all IS (with the exception of 30 mM IS exposure at 12 hours). For 6 hours of exposure, SGSC1512 showed decreased susceptibility to cephalothin only at 3.33 mM IS, whereas at 10 and 30 mM IS the isolates remained with intermediate resistance (Table 1).

The control group of the wild type *S. enterica* ser. Typhimurium strain ST5383 showed full susceptibility to all antibiotics except amoxicillin-clavulanic acid and nalidixic acid, for which only intermediate resistance was observed (Table 2). Interestingly, exposure to GW increased the resistance of ST5383 against nalidixic acid for all conditions except for 12 hours at 30 mM IS. ST5383's intermediate resistance to amoxicillin-clavulanic acid decreased to the susceptible level when it was exposed to GW for 24 hours at all IS and after 18 hours at 10 mM IS (Table 2).

The control group of the wild type and non-motile *S. enterica* ser. Pullorum strain SA1685 was susceptible to all tested antibiotics. This strain did not show any changes in its antibiotic resistance profile and remained susceptible to all antibiotics tested for all experimental conditions as shown in Table 3.

Table 1. Antibiotic susceptibility profile of SGSC1512 exposed to different IS (in mM) GW with residual antibiotics for four different time periods. The letters denote the degree of resistance as S; Susceptible, I; Intermediate Susceptible and R; Resistant.

Antibiotic[a]	Control	6 Hours			12 Hours			18 Hours			24 Hours		
		3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
AMK30[b]	S	S	S	S	S	S	S	S	S	S	S	S	S
AMC30	S	S	S	S	S	S	S	S	S	S	S	S	S
FOX30	S	S	S	S	S	S	S	S	S	S	S	S	S
CRO30	S	S	S	S	S	S	S	S	S	S	S	S	S
KF30	I	S	I	I	S	S	I	S	S	S	S	S	S
C30	S	S	S	S	S	S	S	S	S	S	S	S	S
CIP5	S	S	S	S	S	S	S	S	S	S	S	S	S
NA30	I	I	I	I	I	I	I	I	I	I	I	I	I
TE30	S	S	S	S	S	S	S	S	S	S	S	S	S
SXT25	S	S	S	S	S	S	S	S	S	S	S	S	S

[a]Antibiotic abbreviations are as follows: Amikacin: (AMK); Amoxicillin-clavulanic acid: (AMC); Cefoxitin: (FOX); Ceftriaxone: (CRO); Cephalothin: KF 30; Chloramphenicol: (C); Ciprofloxacin: (CIP); Nalidixic acid: (NA); Tetracycline: (TE); Trimethoprim-sulfamethoxazole: (SXT).
[b]Numbers denote the concentration (in µg) of the given antibiotic impregnated on 6mm disks.

Table 2. Antibiotic susceptibility profile of ST5383 exposed to different IS (in mM) groundwater (GW) with residual antibiotics compared for four different time periods. The letters denote the degree of resistance as S; Susceptible, I; Intermediate Susceptible and R; Resistant.

Antibiotic[a]	Control	6 Hours			12 Hours			18 Hours			24 Hours		
		3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
AMK30[b]	S	S	S	S	S	S	S	S	S	S	S	S	S
AMC30	I	I	I	I	I	I	I	I	S	I	S	S	S
FOX30	S	S	S	S	S	S	S	S	S	S	S	S	S
CRO30	S	S	S	S	S	S	S	S	S	S	S	S	S
KF30	I	I	I	I	I	I	I	I	I	I	I	I	I
C30	S	S	S	S	S	S	S	S	S	S	S	S	S
CIP5	S	S	S	S	S	S	S	S	S	S	S	S	S
NA30	S	I	I	I	I	I	S	I	I	I	I	I	I
TE30	S	S	S	S	S	S	S	S	S	S	S	S	S
SXT25	S	S	S	S	S	S	S	S	S	S	S	S	S

[a]Antibiotic abbreviations are as follows: Amikacin: (AMK); Amoxicillin-clavulanic acid: (AMC); Cefoxitin: (FOX); Ceftriaxone: (CRO); Cephalothin: KF 30; Chloramphenicol: (C); Ciprofloxacin: (CIP); Nalidixic acid: (NA); Tetracycline: (TE); Trimethoprim-sulfamethoxazole: (SXT).
[b]Numbers denote the concentration (in µg) of the given antibiotic impregnated on 6 mm disks.

Table 3. Antibiotic susceptibility profile of SA1685 exposed to different IS (in mM) groundwater (GW) with residual antibiotics compared for four different time periods. The letters denote the degree of resistance as S; Susceptible, I; Intermediate Susceptible and R; Resistant

Antibiotic[a]	Control	6 Hours			12 Hours			18 Hours			24 Hours		
		3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
AMK30[b]	S	S	S	S	S	S	S	S	S	S	S	S	S
AMC30	S	S	S	S	S	S	S	S	S	S	S	S	S
FOX30	S	S	S	S	S	S	S	S	S	S	S	S	S
CRO30	S	S	S	S	S	S	S	S	S	S	S	S	S
KF30	S	S	S	S	S	S	S	S	S	S	S	S	S
C30	S	S	S	S	S	S	S	S	S	S	S	S	S
CIP5	S	S	S	S	S	S	S	S	S	S	S	S	S
NA30	S	S	S	S	S	S	S	S	S	S	S	S	S
TE30	S	S	S	S	S	S	S	S	S	S	S	S	S
SXT25	S	S	S	S	S	S	S	S	S	S	S	S	S

[a]Antibiotic abbreviations are as follows: Amikacin: (AMK); Amoxicillin-clavulanic acid: (AMC); Cefoxitin: (FOX); Ceftriaxone: (CRO); Cephalothin: KF 30; Chloramphenicol: (C); Ciprofloxacin: (CIP); Nalidixic acid: (NA); Tetracycline: (TE); Trimethoprim-sulfamethoxazole: (SXT).
[b]Numbers denote the concentration (in µg) of the given antibiotic impregnated on 6 mm disks.

Epithelial cell invasion assay

The number of CFUs for control groups invading HEp2 cells ranged approximately from 300 to 420 (Fig. 1). After exposure to GW for 6 and 12 hours at all IS tested, the number of all *Salmonella* strains invading the epithelial cells lines decreased significantly. The same trend was also observed after 18 hours of exposure to GW for all strains except ST5383 at 10 mM and 33 mM and SGSC1512 at 30 mM IS. Longer exposure periods (i.e. 24 hours) to GW was associated with an increase in the number of bacteria invading the HEp2 cells at all IS, with the exception of 3.33 mM for SGSC1512 and ST5383. The invasiveness of SA1685 exposed to all IS for 24 hours increased significantly compared to the control group (Fig. 1).

Representative images of SGSC1512 strain invading HEp2 cells are presented in Fig. 2.

Viability of Salmonella under stress

The percentages of viable cells of both the control and test groups of all *Salmonella* isolates exposed to 3.33, 10, and 30 mM GW with residual antibiotics for 6, 12, 18, and 24 hours were given in Fig. 3. As can be seen in Fig. 3, viability values of the control groups were determined to be over 90%. Throughout the spectrum of exposure conditions, residual antibiotics did not cause major viability loss; the lowest viability values observed were approximately 85%, 83% and 86% for SGSC1512, ST5383 and SA1685, respectively.

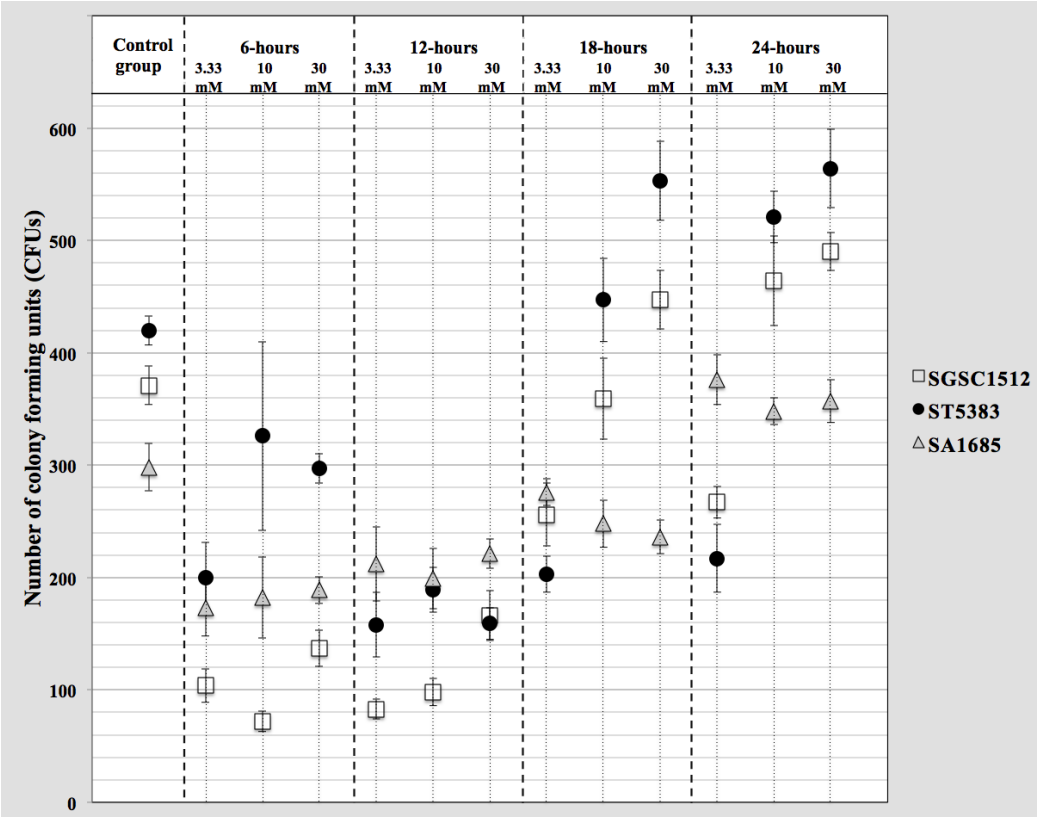

Fig. 1. Changes in the number of *Salmonella* CFUs infecting HEp2 cells upon exposure to GW with residual antibiotics at different IS (in mM) for four different time periods. Data represent average values of three biological replicates and the error bars reflect standard deviations.

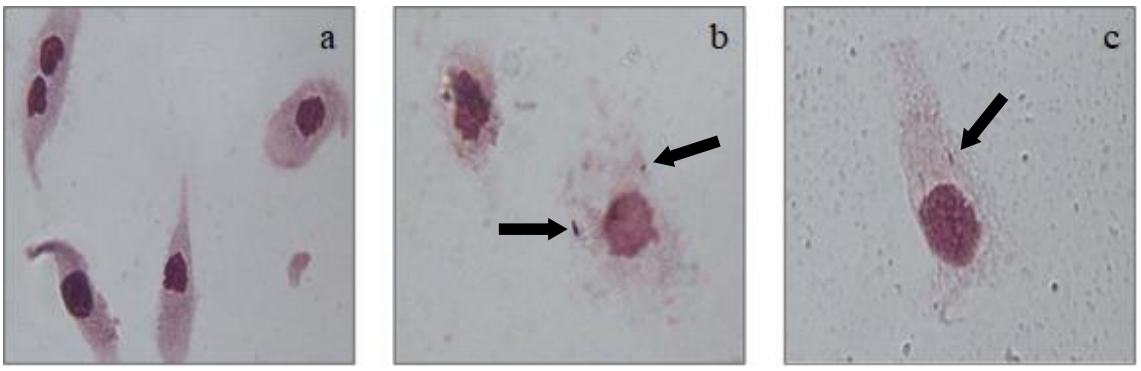

Fig. 2. Representative light microscopy (400×) images of (a) uninfected HEp2 cells and (b,c) HEp2 cells (pointed with the black arrows) infected with SGSC1512.

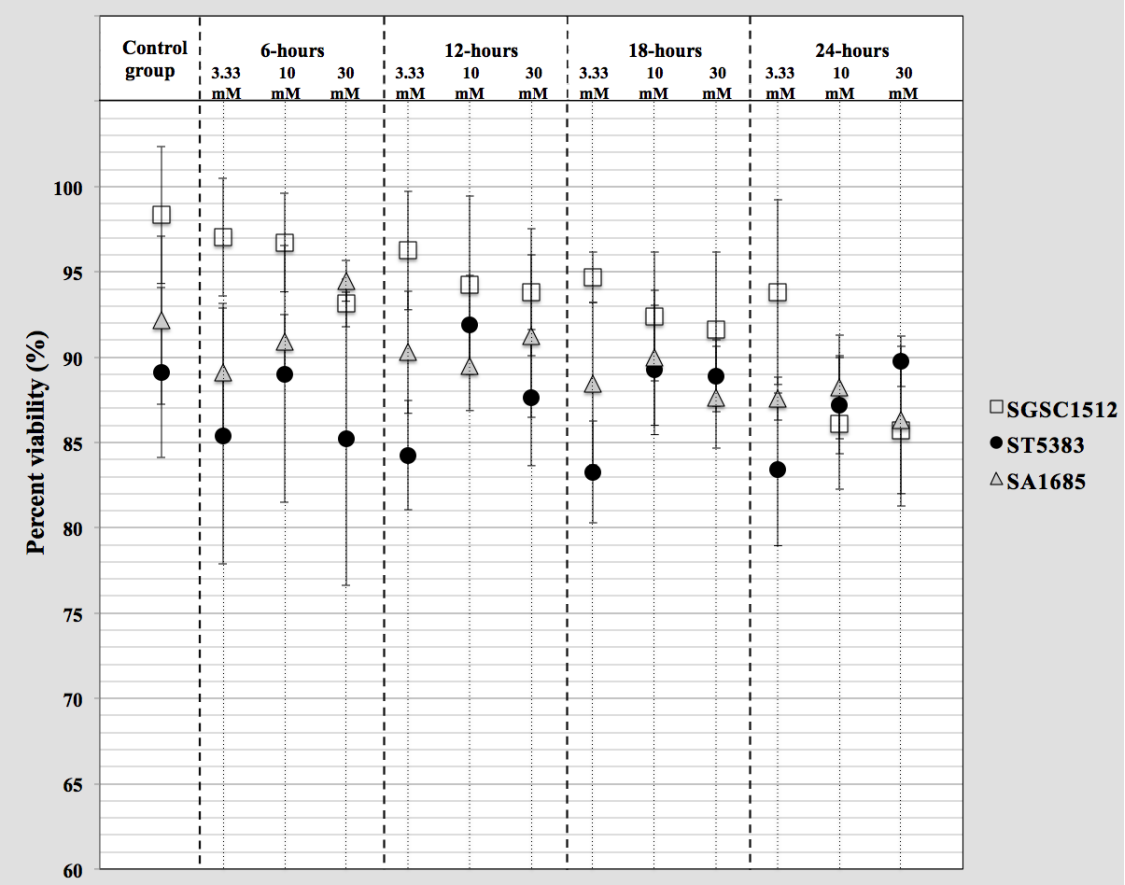

Fig. 3. Changes in the percent viability of *Salmonella* isolates upon exposure to GW with residual antibiotics at different IS (in mM) for four different time periods. Data represent average values of three biological replicates and the error bars reflect standard deviations.

Discussion

In this study, the changes in pathogenicity and viability of outbreak causing *S. enterica* serotypes exposed to groundwater with residual antibiotics were investigated to determine whether this groundwater matrix might impose fitness changes in terms of pathogenicity (invasiveness to target cell lines) and survivability. In addition, different IS conditions were tested to see if any correlation would exist between bacterial efflux pumps (as antibiotic resistance mechanisms) and the external GW environment with varying IS. The results showed increased virulence to be more evident at longer durations (i.e. 24 hours), sustained viability, and strain-dependent decreased antibiotic resistance among *S. enterica* serotypes upon exposure to GW with residual antibiotics. However, no conclusive evidence has been found to link the contribution of efflux pumps to antibiotic resistance of the bacteria under varying IS.

The first organism tested was *S. enterica* ser. Typhimurium strain SGSC1512 with dysfunctional flagella making it a non-motile strain. During exposure to GW, SGSC1512's resistance to cephalothin decreased from an intermediate resistance to a susceptible level for the exposure period of 12 hours and further (with the exception of 30 mM IS exposure at 12 hours). Although cephalothin is a member of the β-lactam class antibiotics, the same trend was not observed for the other β-lactam antibiotics. The influence of efflux pumps (that are likely to be impacted with varying IS of the external environment) on resistance to β-lactams class antibiotics was previously documented in the literature (Lacroix, 1996; Nikaido et al., 1998) but the results of the present study did not confirm this influence. Both the control and the test groups of SGSC1512 showed intermediate levels of susceptibility when tested against nalidixic acid. Nalidixic acid is a quinolone class antibiotic which is normally pumped out of the Gram-negative bacteria cytoplasm via their active efflux pumps. This is in agreement with previous studies showing the effects of efflux pumps mediating reduced quinolone susceptibility in *Salmonella* (Cebrián *et al.* 2005, Chu *et al.* 2005) However, we did not observe an increased resistance in the case of ciprofloxacin, another quinolone antibiotic, most probably due to the slightly different physical and chemical structures of the two drugs, with possible variations in the influx and efflux trends.

The second organism tested was a wild type, outbreak-causing *S. enterica* ser. Typhimurium strain (ST5383). Exposure to GW resulted in increased resistance of the control group from susceptible level to an intermediate resistance for all tested conditions, which was apparent by the statistically significant increased zone diameters at 3.33 mM for 12 hours and at 10 mM for 18 hours against

nalidixic acid. As in the case of resistance profile of SGSC1512, no change was observed against the other quinolone ciprofloxacin for the tested conditions. At longer exposure conditions (i.e. 24 hours for all IS) the intermediate level of resistance of the control group of ST5383 against amoxicillin-clavulanic acid decreased to susceptible levels. The same decrease was also observed at 10 mM GW after 18 hours of exposure. As mentioned earlier, *S. enterica* serotypes shows resistance to β-lactams by the AcrAB efflux system. However, there was no change in the resistance profiles of ST5383 against cefoxitin, ceftriaxone, and cephalothin, all in β-lactam group. This suggest that there is either no direct correlation between the efflux pumps assisting the ST5383 strain to sustain resistance against amoxicillin-clavulanic acid or the fitness of the strain was weakened following the 24 hours GW exposure. Another possibility is the secretion and release of β-lactamases by pathogenic bacteria is a more direct way of mediating the β-lactams. Long-term GW exposure might have negatively impacted the synthesis of β-lactamase in ST5383, which is observed as increased susceptibility.

The last organism tested was *S. enterica* ser. Pullorum strain SA1685, a non-flagellated serovar responsible for avian diseases in the poultry industry. The control group of SA1685 was observed to be susceptible to all antibiotics tested. The exposure of this strain to GW did not cause a change in the antibiotic resistance profile. Since SA1685 is a genotypically and phenotypically different serotype than SGSC1512 and ST5383, this trend might be due to the morphological features and overall fitness of the bacterium and its response to GW stress conditions.

Epithelial cell invasion assay results showed that exposure to GW for 6 and 12 hours resulted in decreased invasiveness of *S. enterica* serotypes into HEp2 cell lines for all tested IS conditions (Fig. 1). In addition, the number of SA1685 that can invade epithelial cells also decreased at all IS for 18 hours of exposure. The number of bacteria that can invade HEp2 cells decreased for 3.33 mM and 10 mM GW, and 3.33 mM for 18 hours of exposure in the cases of SGSC1512 and ST5383, respectively. However, at 24 hours of exposure to GW, increased number of ST5383 and SGSC1512 that can invade the epithelial cells were observed at 10 and 30 mM IS. In the case of SA1685, the number of invading CFU increased for all tested IS for 24 hours of exposure. The decreases in the number of invading bacteria observed up to 18 hours of exposure might be related to the decreased fitness induced by the residual antibiotics in the GW. After 24 hours of exposure, the bacteria might have metabolically become accustomed to the GW matrix, which in return increased the number of invading bacteria. Another possibility is that prolonged exposure to GW with divalent cations such as Ca^{2+} in this case may have contributed to the increased number of CFUs infecting HEp2 cells, which is in parallel to previous studies that showed increased invasion of human cell lines with increased Ca^{2+} concentrations (Niesel & Peterson 1987, Peterson 1988).

In conclusion, although the occurrence of residual antibiotics in groundwater systems is common, there is very limited knowledge on the effects of these antibiotics on the fitness levels of pathogenic bacteria with respect to their pathogenicity and survivability. In this initial study, outbreak-causing *S. enterica* serotypes with different morphologies were exposed to groundwater conditions with residual antibiotics to test for any changes in invasiveness against human cell lines. Their viabilities were also monitored. In addition, as an ion-rich environment, groundwater may affect the active efflux pumps of bacteria, which in turn change their resistance profiles against certain antibiotic classes. The results showed that relatively short-term exposure to residual antibiotics at μg per liter levels will not decrease viabilities, but may still induce increased invasiveness of *S. enterica* serotypes into human epithelial cell lines, which was more evident at 24 hours of exposure in the present conditions. Although there was no clear evidence to link the efflux pump mediated antibiotic resistance to different concentrations of ions tested in this study, subtle changes in resistance profiles were observed in certain quinolone and β-lactam class antibiotics. Further analyses might elaborate these findings to investigate the fate of *S. enterica* serotypes in groundwater settings.

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