Journal of Pollution Effects & Control
ISSN: 2375-4397
Al Yousef et al., J Pollut Eff Cont 2016, 4:1
DOI: 10.4172/2375-4397.1000155
Research Article
Open Access

Detection of Extended Spectrum Beta-Lactamase Producing *Escherichia Coli* on Water at Hafr Al Batin, Saudi Arabia

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Abstract

In this study antibiotic resistant *Escherichia coli* from household water collected in Hafr AlBatin, Saudi Arabia, were characterized by prevalence of extended spectrum betalactamase (ESBL). Samples were collected from drinking and washing water at 12 locations. From the 144 samples obtained, Millipore membrane filters incubated on trypton soya agar plates produced colonies that yielded 34 isolates of *E. coli* as verified by biochemical tests. Isolates suspected ESBL producing were tested by using MicroScan analysis and disc diffusion test as ESBL phenotypic confirmatory methods. Phenotypically confirmed ESBL isolates were examined for antimicrobial susceptibility against 30 antibiotics and amplification of bla_{VEM}, bla_{CTX}, bla_{TEM}, bla_{GES} and bla_{SHV} genes by polymerase chain reaction. Out of 34 *E. coli* isolates, only 6 (17.6%) were positive for ESBL producing according to MicroScan analysis. Disk diffusion as a confirmatory test indicates sensitivity of MicroScan system. PCR results indicated that the VEB was the most prevalent (83.3%) followed by CTX gene (16.6%) between these isolates. The ESBL-producing *E. coli* isolates was fully susceptible to pip/tazo (100%) and fully resistance to ampicillin, cefazolin and pepracillin (100%). This study showed that ESBL-producing *E. coli* are multidrug-resistant and existent in Hafr Al Batin's water. Also, data indicated that wastewater maybe contributes as a source and reservoir of antibiotic resistance.

Keywords: Antimicrobial resistance; ESBL; *E. coli*; Water

Introduction

One of the major ways of diffusion of pathogenic and/or antibiotic resistant bacteria, through water, soil and air. Multidrug resistant bacteria have been revealed in different water sources including rivers, lakes, groundwater and drinking water [1-4]. Water consumption, can command to habitation of the gastrointestinal tract of humans and animals by bacteria containing resistance genes and barter genes with bacteria previously present in the intestinal tract [5,6].

Some bacteria can make beta-lactamase enzymes that cleave the beta-lactam ring and then disrupt the antimicrobial action [7]. An extended spectrum beta-lactamase (ESBL)-producing bacteria is capable to disrupt the third generation cephalosporins and monobactum [8]. Also, shown to be able to combat quinolones [9]. ESBL-producing bacteria especially of Enterobacteriaceae, foremost *E. coli* and *Klebsiella pneumonia* [10]. ESBL-producing *E. coli* have 3 various resistance mechanisms. The most widespread is production of beta-lactamase which hydrolyzes the beta-lactam ring in penicillins and cephalosporins. The second is mutation which decrease beta-lactam uptake [11]. The third resistance mechanism is existence of efflux pumps, which exports antibiotics outside of the cell.

Beta-lactam antibiotics work by prevents cell wall biosynthesis in the bacteria. The most common reason of resistance to beta-lactam antibiotics like penicillin is production of enzymes named beta-lactamases. Beta-lactamases are a family of enzymes produced by numerous of Gram positive and negative bacteria that disrupt beta-lactam antibiotics by slit the beta-lactam ring [12].

Penicillins and concerned antibiotics have been utilized extensively for the control and treatment of bacterial infections. Efficiency of this group of antimicrobial agents has been amended, because of the evolution of multidrug-resistant strains of bacteria [13]. Through the years, incalculable penicillin derivatives [14] have been designed and tested, and a variety of new β-lactam ring systems have been introduced such as cephalosporins, cephamycins, oxacepems, clavulanic acid and carbapenems.

Saudi Arabia with an area of 2.15 million km^2 is a desert and water deficit country, with limited fresh water-supplies. It is also recognized by low annual rainfall and privation perennial rivers. The water resources in the Saudi Arabia are surface and underground deposits. Water collected through rainfall (surface water).

In Hafr Al Batin city about 100% of water supply comes from groundwater. Water transported by car to the house where stored in the wells dedicated to it, which may be located next to the sewage tanks. No available information about extended-spectrum beta-lactamases (ESBLs) producing bacteria in water Hafr Al Batin. The present study was to gain insight into the prevalence of AMR *E. coli* in washing and drinking water, and to check the possible role of sewage tanks as AMR contamination source of water.

Materials and Methods

Water sampling

Water samples were taken from 12 locations in the Hafr Al-Batin city, Saudi Arabia (Figure 1). Three sampling sites were located on each location. Two of which were from washing water that taken from house tanks in most of residential neighborhoods and one from drinking water from charity refrigerators. Sampling was done twice, the first on January (winter) and the second on July (summer). Two samples were collected each time at each collection site at two different times, and we

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Received January 25, 2016; **Accepted** February 24, 2016; **Published** February 29, 2016

Citation: Al Yousef SA, Farrag ES, Ali AM, Mahmoud SY (2016) Detection of Extended Spectrum Beta-Lactamase Producing *Escherichia Coli* on Water at Hafr Al Batin, Saudi Arabia. J Pollut Eff Cont 4: 155. doi:10.4172/2375-4397.1000155

Figure 1: Location map of the study area of Hafr Al-Batin city, Saudi Arabia.

used three replicates from each sample for tests. Collected samples were transported by standard methods as mentioned in APHA, 1989 [15].

Isolation of *E. coli*

100 mL water sample is filtered through 0.45 µm pore size membrane filters (Millipore, the Netherlands). Filters were incubated on tryptone soya agar (TSA) at 36 ± 2°C during 4-5 hours, and subsequently transferred to tryptone bile agar (TBA) then incubated for 19-20 hours at 44 ± 0.5°C as described by Sing et al. [16]. *E. coli* identified using the TSA/TBA method (i.e. indole-positive), were supplementary confirmed as *E. coli* by testing for ß-glucuronidase-activity on Brilliance *E. coli*/coliform agar. Beta-glucuronidase-positive colonies identified using tryptone bile x-glucuronide agar (TBX) was extra confirmed by MicroScan [17] for identification and antibiotic susceptibility.

Analysis of antimicrobial resistance

Generally, 99 *E. coli* isolates (91 from washing water and 8 from drinking water) were obtained. These were screened for confirmation of identification and its susceptibility to a panel of antimicrobials of human and veterinary clinical relevance, using MicroScan. MicroScan® instrumentation (auto SCAN®-4 and WalkAway® System) (Siemens Healthcare Diagnostics Inc, USA) was used. Panels used were MicroScan Dried Gram Positive MIC/Combo, Dried Gram Positive Breakpoint Combo and Dried Gram Positive ID Type 2 or 3. Also, MicroScan Dried Gram Negative MIC/Combo panels and Dried Gram Negative Breakpoint Combo Panels were used. MicroScan panels were designed for use in determining agent susceptibility and/or identification to the species level of rapidly growing aerobic and facultative Gram positive cocci or aerobic and facultatively anaerobic Gram negative bacilli. MICs obtained for ceftazidime and cefotaxime with CA were compared with those obtained with the same drugs without CA. Subsequently, strains were considered as ESBL-positive or -negative in accordance with CLSI recommendations (CLSI, 2010). The tests were performed as recommended by supplier guidelines [17]. Susceptible, intermediate and resistant isolates were arranged according to antibiogram results.

Multi-drug resistance was defined as resistance to 3 or more different classes of antimicrobials according to CLSI, 2010 [18].

Confirmation of ESBL-producing *E. coli*

Suspected ESBL-*E. coli* isolates (after MicroScan analysis) were confirmed for ESBL-production by double disc synergy test (DDST) according to CLSI guidelines (CLSI, 2010) [18]. DDS test results analysis. Susceptibility testing was performed (McFarland 0.5 standard) on Mueller–Hinton agar (MHA) by placing discs on the agar surface containing 30 mg cefotaxime or ceftazidime, with and without 10 mg CA. Plates were incubated at 37°C for 24 h. According to CLSI guidelines, strains were considered positive for ESBL production whenever zone diameters increased by ¢5 mm for cefotaxime or ceftazidime when tested in combination with CA. This method was considered the gold standard for method comparison.

DNA isolation and genotyping

A single colony from each ESBL-producing isolate was transferred into 100 µL of sterile distilled water and the bacterial DNA was extracted by using boiling method included microwave pre-heating according to Ahmed et al. [19]. PCR screening for presence of different beta-lactamase genes was performed. PCR was carried out and specific primers (Table 1) were used for VEB, CTX, TEM and GES genes. PCR mixtures were prepared by using 5 µL template DNA (about 500 pg of DNA), 12.5µL PCR master mix; 1 × PCR buffer [Tris-Cl, KCl, $(NH_4)2SO_4$, 1.5mM $MgCl_2$] (pH 8.7), 200 µM dNTP, and 1 µL of each 10 pM primer and 0.5U Taq DNA polymerase in a final volume of 25 µL. The amplification reaction was carried out in a Thermal Cycler (Eppendorf master cycler®, MA) with an initial denaturation (94°C for five minutes) followed by 30 cycles of denaturation (94°C for 25 seconds) annealing (52°C for 40 seconds), and extension (72°C for 50 seconds) and a single final extension at 72°C for six minutes [20]. The amplified products were electrophoresed on 2% agarose gel and visualized on a gel document system after staining with ethidium bromide (0.5 mg/mL). A non-ESBL-producing strain (*E. coli* ATCC 25922) was used as a negative control.

Results and Discussion

Water borne infections still ravage the global community and are responsible for millions of deaths per year. Water that looks clear and pure may be sufficiently contaminated with pathogenic microorganisms to be a health hazard.

ESBL-producing *E. coli*

Ninety-nine *E. coli* isolates were detected and isolated from the water samples using the TSA/TBA method. Only 34 isolates were confirmed as *E. coli* by MicroScan. ESBL-producing strains (6/34) were found in all the samples analyzed by MicroScan system (Table 2). Five ESBL-producing *E. coli* were detected from the washing water samples at five locations (Al-Khalediyah, Al-Rabwah, Al-Rawdhah, Al-Nayefiyah and Al-Nakheel) at summer season only, no winter was found. On the other hand, ESBL-producing *E. coli* was detected and isolated from drinking water in one location (Al-Baladiyah) out of 12.

Genes	Primer used (5'-3')	PCR product size
VEB	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	585 bp
CTX	CAAAGAGAGTGCAACGGATG ATTGGAAAGCGTTCATCACC	205 bp
TEM	TAATCAGTGAGGCACCTATCTC GAGTATTCAACATTTCCGTGTC	800 bp
GES	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	846 bp
SHV	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	867 bp

Table 1: The sequences of primers used in PCR amplification of beta-lactamase genes.

Location	Season	Washing water isolates		Drinking water isolates		Total/ confirmed (%)
		Total	+	Total	+	
Abu-Musa al-Asha'ari	Winter Summer	1 4	0 0	0 0	0 0	1/0 (0.0) 4/0 (0.0)
Al-Aziziah	Winter Summer	0 2	0 1	0 0	0 0	0/0 (0.0) 2/1 (50.0)
Al-Khalediyah	Winter Summer	1 2	0 0	0 0	0 0	1/0 (0.0) 2/0 (0.0)
Al-Rabwah	Winter Summer	0 1	0 1	0 1	0 0	0/0 (0.0) 2/1 (50.0)
Al-Muhammadiyah	Winter Summer	0 3	0 0	0 0	0 0	0/0 (0.0) 3/0 (0.0)
Al-Baladiyah	Winter Summer	1 3	0 0	0 2	0 1	1/0 (0.0) 5/1 (20.0)
Al-Rawdhah	Winter Summer	0 4	0 1	0 0	0 0	0/0 (0.0) 4/1 (25.0)
Al-Nayefiyah	Winter Summer	0 1	0 1	0 0	0 0	0/0 (0.0) 1/1 (100.0)
Al-Sulaimaniyah	Winter Summer	0 0	0 0	0 0	0 0	0/0 (0.0 0/0 (0.0)
Al-Faisaliyah	Winter Summer	0 1	0 0	0 0	0 0	0/0 (0.0) 1/0 (0.0)
Al-Masyef	Winter Summer	0 4	0 0	0 0	0 0	0/0 (0.0) 4/0 (0.0)
Al-Nakheel	Winter Summer	0 2	0 1	0 1	0 0	0/0 (0.0) 3/1 (33.3)
Total		30	5	4	1	34/6 (17.6)

Table 2: Total number and ESBL-*E. coli* isolates, which confirmed (+) by MicroScan system from 13 locations.

The prevalence of ESBL-producing *E. coli* varied between waters that differed with regard to region, type of water, and time of the year at sampling. Perhaps, prevalence may vary with the number of faecal dirtiness sources in the neighborhood of sampling sites and factors affecting when and how often releasing takes place (climate or season). Our results agreed with Blaak et al. [21]; Adnan et al. [22].

All ESBL-producing isolates also showed resistance to other antimicrobials: 100% to ampicillin, cefazolin, cefepime, cfuroxime, mezlocillin, piperacillin, trimethoprime, trimethoprime/sulfamethoxazole; 83.4% to ciprofloxacin, gentamicin, levofloxacin, norfioxacin, tetracycline, tobramycin; 50% to cefoxitin; 33.3% to ertapenem and 16.6% to fosfomycin, imipenem, meropenem, nitrofurantain, tigecycline (Table 3). The study by Babypadmini and Appalaraju [23] reported 74% resistance to trimethoprim/sulfamethoxazole in ESBL-producing *E. coli* pathogens by disk diffusion method, which is lower than our results. This difference may be due to use of different methods of evaluation for determining the susceptibility. We determined the antimicrobial resistance by the MicroScan method which is more sensitive than disk diffusion method.

Validation of ESBL-producing isolates

ESBL confirmation test was carried out by disk diffusion method. For validity testing of 6 *E. coli* ESBL- producing isolates, no errors were showed between the two test methods, MicroScan and disk diffusion test.

ESBL genes

VEB and CTX-M genes were detected in 5 and 1 ESBL-producing

Antibiotics	Washing water-*E. coli* isolates										Drinking water-*E. coli* isolate	
	1		2		3		4		5		MIC	Interps
	MIC	Interps	MIC	Interps	MIC	Interps	MIC	Interps	MIC	Interps		
Amikacin	≤16	S	>32	R	≤16	S	≤16	S	≤16	S	>32	R
Amox/K Clav	>16/8	R	>16/8	R	≤8/4	S	16/8	I	16/8	I	>16/8	R
Ampicillin	>16	R	>16	R	>16	R	>16	R	>16	R	>16	R
Cefazolin	>16	R	>16	R	>16	R	>16	R	>16	R	>16	R
Cefepime	>16	R	>16	R	>16	R	>16	R	>16	R	>16	R
Cefotaxime	>32	ESBL	>32	ESBL	>32	ESBL	>32	ESBL	>32	ESBL	>32	ESBL
Cefotaxime/K Clav.	≤0.5		≤0.5		≤0.5		≤0.5		≤0.5		≤0.5	
Cefoxitin	≤8	S	>8	R	≤8	S	≤8	S	>8	R	>8	R
Ceftazidime	>16	ESBL	>16	ESBL	>16	ESBL	>16	ESBL	>16	ESBL	>16	ESBL
Ceftazidime/k clav.	2		2				≤0.25		≤0.25		2	
Cefuroxime	>16	R	>16	R	>16	R	>16	R	>16	R	>16	R
Ciprofloxacin	>2	R	>2	R	≤1	S	>2	R	>2	R	>2	R
Colistin	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S	>4	R
Ertapenem	≤2	S	>4	R	≤2	S	≤2	S	≤2	S	>4	R
Fosfomycin	≤32	S	≤32	S	≤32	S	≤32	S	≤32	S	>32	R
Gentamicin	>8	R	>8	R	≤4	S	>8	R	>8	R	>8	R
Imipenem	≤4	S	≤4	S	≤4	S	≤4	S	≤4	S	>8	R
Levofloxacin	>4	R	>4	R	≤2	S	>4	R	>4	R	>4	R
Meropenem	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	>8	R
Mezlocillin	>64	R	>64	R	>64	R	>64	R	>64	R	>64	R
Moxifloxacin	>1	R	>1	R	1	I	>1	R	>1	R	>1	R
Nitrofurantoin	≤32	S	≤32	S	≤32	S	≤32	S	≤32	S	>64	R
Norfioxacin	>8	R	>8	R	>4	S	>8	R	>8	R	>8	R
Pip/tazo	64	I	≤16	S	≤16	S	≤16	S	≤16	S	≤16	S
Piperacillin	>64	R	>64	R	>64	R	>64	R	>64	R	>64	R
Tetracycline	>8	R	>8	R	≤	S	>8	R	>8	R	>8	R
Tigecycline	≤1	S	≤1	S	≤1	S	≤1	S	≤1	S	>2	R
Tobramycin	>8	R	>8	R	≤4	S	>8	R	>8	R	>8	R
Trimeth/sulfa	>2/38	R	>2/38	R	>2/38	R	>2/38	R	>2/38	R	>2/38	R
Trimethoprim	>8	R	>8	R	>8	R	>8	R	>8	R	>8	R

S = Susceptible, I = Intermediate, R = Resistant, MIC = Minimum Inhibitory Concentration (mcg/m), Interps = Interpretation,
ESBL = Extended spectrum beta-lactamase. Suspected ESBL (confirmatory test needed to differentiate ESBL from other beta-lactamase)

Table 3: Antibiotic susceptibility pattern among ESBL-*E. coli* resistant isolates.

E. coli isolates from washing and drinking water samples, respectively. The results of ESBL genotyping showed that VEB gene was the most prevalent (83.3%) followed by CTX gene (16.6%), TEM (0.0%), GES (0.0%) and SHV (0.0%). ESBL-producing *E. coli* isolate from drinking water was carried CTX gene (Figures 2 and 3). In washing water, only VEB gene was predominant. VEB was the most frequent resistant gene in ESBL-producing *E. coli* isolates in this study. The study by Rezai et al. [24] reported that TEM resistant gene was most prevalent. TEM,

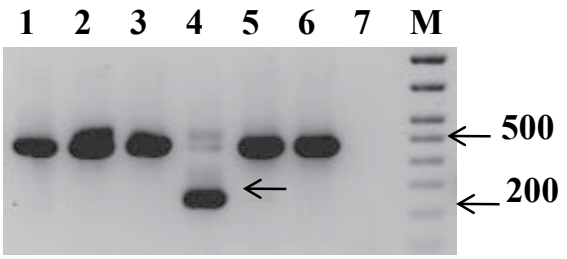

Figure 2: Agarose gel showing the 585 and 205bp PCR fragments band for VEB and CTX genes from ESBL-producing *E. coli* isolates, respectivily. Lanes: M: molecular weight marker; 1, 2, 3, 5 and 6: VEM positive washing water isolates; 4: CTX positive drinking water isolate and 7: negative control.

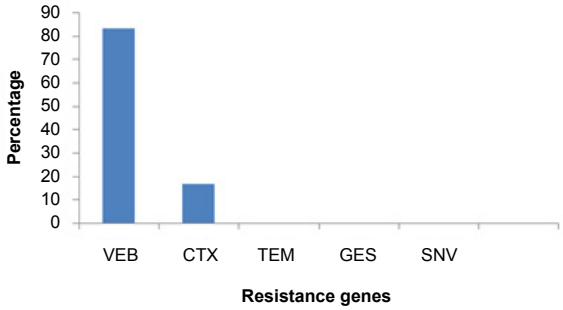

Figure 3: Distribution of resistance genes in ESBL-producing *E. coli* isolates.

GES and SHV resistant genes were not found in ESBL-producing *E. coli* isolates which is in line with the low frequency of this gene in ESBL-producing *E. coli* strains [25].

Conclusion

Fecal contamination of tank water in Hafr Al Batin city by sewage may be occurs through discharge of untreated sewage through sewage leaking or during heavy rainfall. Water distribution and storage

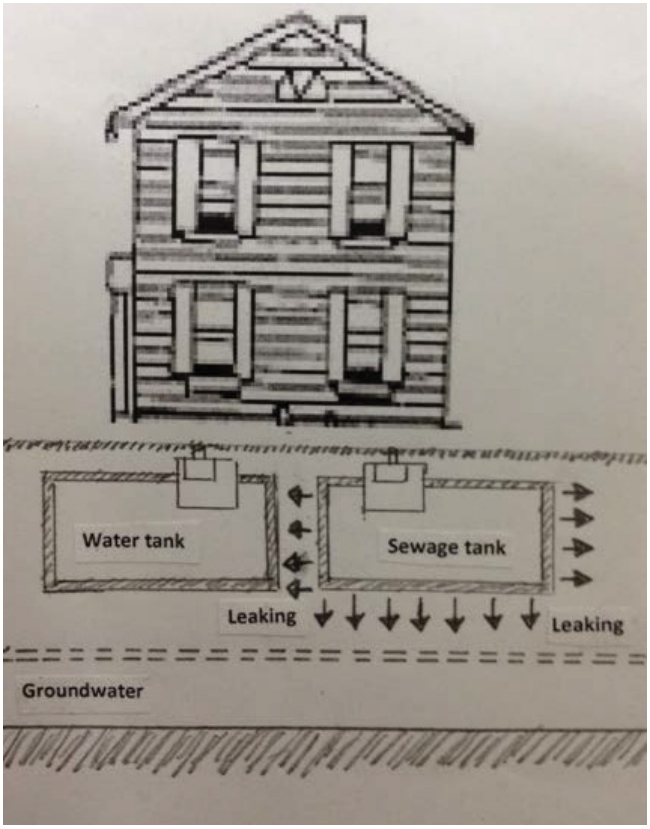

Figure 4: Diagram showing pathway of contaminants emission from sewage tank to adjacent water tank and groundwater.

systems in Hafr Al-Batin city could serve as an incubator for growth of certain ARB populations and as an important reservoir for the spread of antibiotic resistance to opportunistic pathogens. Out of six ESBL-producing *E. coli* isolates, five were obtained from washing water tanks adjacent to sewage tanks as shown in proposed pathway of contamination transport (Figure 4). Leaks happen when a pipe isn't sealed in a specific spot. Most old septic tanks didn't have any sealants applied to the mating joints and troubleshooting difficult. Also, heavy vehicles can crush septic system drain lines causing leakage and toxic smells and odors. The septic cover can cause leaks because they may not be well-secured. From here, we recommend that the system of quantitative and qualitative microbial risk estimate must be applied on houses water tanks at Hafr Al-batin city. This process was successfully implemented for estimate exposure and infection hazard of bacterial and other pathogens from consumption of drinking water and washing water. Water-borne transmission has been demonstrated to be a relevant route of transmission for faecal bacterial species, including *Salmonella* and *E. coli* [26,27]. To evaluate risks of human exposure, the system of quantitative microbial risk assessment could be used [28]. This process was successfully implemented for estimate exposure and infection hazard of bacterial and other pathogens from consumption of drinking water and recreational water [29,30].

Our results suggested the possible emissions of the ESBL-producing *E. coli* from sewage tanks to the environment. Water distribution systems in Hafr Al-Batin city could serve as an incubator for growth of certain ARB populations and as an important reservoir for the spread of antibiotic resistance to opportunistic pathogens.

Acknowledgment

The authors extend their appreciation to the Deanship of Scientific Research at University of Dammam for funding this work through research project no. 2014027.

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