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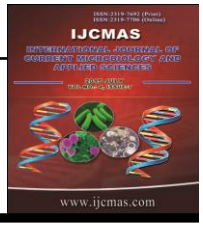

Original Research Article

Microbiological quality of water offered to livestock assessed by *Escherichia coli* and isolates analysis for potential virulence markers and antibiotic resistance

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Keywords

Thermotolernt *E. coli*, Trough water, Virulence genes, Antibiotic resistance, Fecal coliforms

A B S T R A C T

An adequate and safe water supply is essential to the production and health of livestock and poultry. Animal drinking water were analyzed for fecal coliforms and *Escherichia coli* harboring virulence-related genes. Further, isolates were analyzed for their resistance to antibiotics. Animal drinking water (n = 54) collected from water troughs on loose animal houses distributed in Taif region, Saudi Arabia. Water samples were analyzed for fecal coliforms by most probable number and *Escherichia coli* harboring virulence-related genes were assessed by multiples polymerase chain reaction. Further, isolates were analyzed for their resistance to antibiotics by disk diffusion method. A total of 23 (42.6%) thermotolerant *E. coli* were recovered and assigned to group A (4 strains), B1 (12 strains), B2 (4 strains) and D (3 strains). Seven (30.4%) of *E. coli* isolates had one or more genes associated with pathogenicity. The virulence gene signatures encoding for Shigatoxigenic *E. coli* (STEC) were confirmed in four isolates. The intimin coding gene marker (*eae*A) was detected in one isolates. Virulence encoding genes cnf_1 and *cdt*B were indicated in two strains. Antibiogram analysis of the isolates indicated that 43.5% of *E. coli* isolates exhibited resistance to the one or more antimicrobial agents. Multidrug resistance was observed in 17.4% of *E. coli* isolates. The study reported the occurrence of virulence associated genes and resistance to antimicrobial agents in *E. coli* from troughs water and so their possible effects on animal health and productivity.

Introduction

Water is the single most important nutrient for livestock. A safe water supply is essential for essential for optimal livestock health and maximum production of milk and meat. In Saudi Arabia, groundwater sources are the major source of the water supply either for agricultural or animal drinking purposes, therefore its protection is important both socially and economically. As the population growth and interactions increased, by the way lead to widespread contamination of the groundwater by domestic and industrial effluents (Mazari-Hiriart *et al*., 2005).

Contaminated water supplies for livestock and poultry can also contaminate human drinking water. For these reasons, farm water supplies should be protected against contamination from bacteria. livestock drinking water heavily contaminated with enteric bacteria could serve as a common source of exposure to potential pathogens to cattle that could result in infection of large numbers of animals. Fecal coliform bacteria, particularly *E. coli*, is the most commonly used bioindicators of fecal pollution in water and food (Harwood *et al*., 2000). Previous studies have focused mostly on the importance of clinical *E. coli* isolates as an etiological agents of intestinal and extraintestinal infections of human and animals, however, very little is known about their occurrence and pathogenic potential in aquatic environments (Griffin and Tauxe, 1991; Armstrong *et al*., 1996; Osek, 2000).

Laboratory diagnostic examination of water supply may be necessary for maximum animal health. Recently, various PCR-based assays have been developed for the detection of pathogens such as *E. coli* (McDaniels *et al*., 1996; Meng *et al*., 1997; Hu *et al*., 1999; Fratamico *et al*., 2000; Osek 2000). Among these PCR assays multiplex PCR (mPCR) provide a more sophisticated approach, enabling a simultaneous and specific detection of more than one specific species (De Vos *et al*., 1997; Tsen and Jian, 1998; Osek 2000).

The purpose of the present study was to describe the microbiological quality of water commonly present on farms using *E. coli* and further the pathogenic potential and bioresistance of these isolates were assessed.

Materials and Methods

Water Sampling: A simple random sample of 54 groundwater for livestock drinking were collected from water troughs located on different farms within the Taif region, Western Saudi Arabia. Water and sediments were collected from the bottom of each trough in sterile plastic bags and transported to the laboratory on ice within 24 h.

Enumeration of coliforms and *E. coli* isolation: The examination of the water samples using standard coliform Multiple-tube (MPN) fermentation techniques (Clesceri *et al*., 1998). The isolation of Fecal *E. coli* was achieved in water samples by monitoring the acidification and gas production during growth in MacConky broth (Oxoid, UK) at 44±0.5°C for 24±3 h. From the fermentation tube, further recovered on eosin methylene blue agar (Scharlau, Spain, EU) as metallic sheen. The identification of *E. coli* was confirmed by performing API identification strips (bioMerieux-France). Bacterial glycerol stocks were prepared using two aliquots of the bacterial suspension (0.5 mL) diluted with 0.5 mL of 2X tryptic soy broth (TSB - Oxoid) containing 20% glycerol and stored at -70ºC.

Antimicrobial susceptibility and multidrug resistance: The antibiotic sensitivity of the environmental strains was tested with the disc diffusion method on Muller-Hinton agar (Hi-Media, India) using commercial antibiotics (µg/disc). The isolates were screened for resistance to Ampicillin (AMP 25), amoxicillin/ clavulanic acid (AMC 20/10), Nalidixic acid (Nal 30), Streptomycin (STR 10), Ciprofloxacin (CIP 10), Chloramphenicol (CHL 30), Cefotaxime (CTX 30), Ceftazidim (CAZ 30), Cefalothin (CEF 30), Gentamicin (GEM 10), Tetracycline (TET 30), sulfamethoxazole/trimethoprim (SXT 23.75/1.25). All discs were purchased from Hi-Media, India and Mast-Diagnostics, United Kingdome. Few colonies of each isolate were dispensed in sterile normal

saline to match the 0.5 McFarland standards for sensitivity tests as described by National Committee for Clinical laboratory approved Standards (CLSI, 2013).

Molecular biology

DNA Extraction and PCR based confirmation of *E. coli* isolates: Genomic DNA was isolated from *E. coli* by boiling method (Solberg *et al*., 2006). An overnight bacterial culture (200 *µl*) was mixed with 800 *µl* of distilled water, boiled for 10 min and, after cooling, centrifuged and the supernatant was used as the DNA template for the PCR. For confirmation of *E. coli*, PCR was performed by targeting universal stress protein A gene *uspA*, using primer and PCR conditions as previously described (Chen and Griffiths, 1998).

Phylogenetic analysis: A triplex PCR was performed to segregate each isolate into one of the four main phylogenetic groups (A, B1, B2 and D) by targeting two marker genes (*chu*A and *yja*A) and a DNA fragment TSPE4.C2. PCR conditions were same as described earlier by by Clermont *et al*., (2000).

Targeted virulence related genes: There are many virulence genes associated with *E. coli*. We selected some most reported virulence genes. Multiplex PCR was performed for each isolate for the identification of virulence related genes.

Gene regions coding for the following pathogenic properties were amplified for each bacterial isolate: Shiga-like Toxin 1 and 2 (*stx1*, *stx2*), enteropathogenic attachment and effacement (*eaeA*), heat-labile toxin (*LT*), heat-stable toxin (*ST*), cytotoxic necrotizing factor 1 (*cnf*1), cytolethal distending factor (*cdt*B), P-fimbrial (*pap*A), S-fimbrial adhesions (*sfa*S) and alpha haemolysin (*hly*D) using specific primers (Table 1). The amplification was done in a 25-µl containing template DNA, 4 *m*M MgCl2, 0.8 *m*M each of dNTPs, 1 U of Taq polymerase. The PCR assays, specific primer sequences and the predicted size of the amplified products for the different pathogenic gene coding regions were employed as previously described (Brian *et al*., 1992; Heuvelink *et al*., 1995; Johnson and Stell, 2000; Lang lee *et al*., 1994; Schultz *et al*., 1994). For cycling, a *PXE*-0.5 thermal cycler (THERMO, Electron Corporation, Milford, MA, USA) was used. The amplified products were visualized by ethidium bromide staining after gel electrophoresis of 10µl of the final reaction mixture in 1.5% agarose. Reference strains representing the amplified genes, J96 (*pap*A, *hly*D, *cnf*1); JP6 (*cdt*B); Bl-6-9 (*sfa*S) (kindly provided by Prof. JOHNSON & Dr. STELL, VA Medical Centre and Department of Medicine, University of Minnesota, Minneapolis, Minnesota, USA); *E. coli* 26 (stx_1, stx_2, *eae*A) (provided by Bundesst. bakt. serol. Untersuchngsanstalt, National reference laboratory for EHEC, Innsbruck, Austria); for *LT* and *ST* (Prof. AWAD-MASALMEH, Department of 2nd Medical Clinic, University of Veterinary Medicine, Vienna, Austria).

Detection of integrons: All isolates showed antibiotic resistance were tested for the presence of conserved integrons of classes 1, 2 and 3 by using previously described PCR protocol with degenerated primers (White *et al*., 2001).

Statistical analysis: Analysis of variance was used to compare the prevalence of various phylogenetic groups and presence of different virulence related genes. The difference having P value equal or less than 0.05 was considered as statistically significant.

Results and Discussion

Microbial quality and confirmation of *E. coli* isolates: In this pilot investigation of animal drinking water quality, a fairly high fecal coliform pollution displayed according to the results of fermentive growth in MacConkey broth at 44.5 °C, with multiple fermentation test (MPN/100ml) ranged from < 1 to 1100 MPN/100 ml. Of a total of 54 water samples collected, 23 (42.6%) samples were found to recover thermotolerant *E. coli* (Table 2). A total of 23 isolates were confirmed as *E. coli* by conventional biochemical and the specie-specific primer employing PCR assay targeting gene encoding the universal stress protein A (*uspA*). This PCR generates the 884-bp *E. coli*-specific product (Fig 1).

Phylogenetic analysis: Out of 23 *E. coli* isolates, the group B 1 isolates 12 (52.2%) were found significantly higher ($P < 0.05$) as compared to other phylogenetic groups. Three (13.04%) isolates fell in group D and 4 isolates each (17.4%) belonged to group A and B2 (Table 3).

Prevalence of virulence genes among water *E. coli* isolates The targeted virulence genes were detected in 7 (30.4%) isolates. Remaining isolates were negative for the targeted genes. Four of the 11 group B1 isolates showed presence of *stx*-virulence genes while one of group A isolates showed presence of *eae*A-virulence gene. two of group B2 isolates demonstrate the presence of cnf_1 and *cdt*B genes of extraintestinal pathogenic *E. coli* (ExPEC). During the screening, none of the tested *E. coli* isolates could reacted positively with the LT and ST primers coding for enterotoxigenic *E. coli* (ETEC). A representative gel electrophoresis profile of amplified products of some strains is shown in Fig. 2.

Antibiogram analysis and detection of integrons: Of 23 *E. coli* strains recovered, 10 (43.5%) strains exhibited resistant to one or more types of antibiotic. Resistance to various antimicrobial agents was presented in Table 4. Multi-drug resistance (three or more) was observed in four isolates (Table 5). No integrons were detected in the water isolates showing antibiotic resistance.

The quality of water offered to production animals is of vital importance. In Saudi Arabia, increased agricultural and animal production are reliant on groundwater sources. Water that adversely affects the health and growth of animals cannot be considered suitable. The farm water supplies, either surface or ground, should be protected against contamination from microorganisms and other pollutants (Runyan and Bader, 1995).

The presence of coliform bacteria and *E. coli* is an indication for water contamination. Fecal coliform bacteria are the most commonly used bioindicators of fecal pollution in water and food (Harwood *et al*., 2000). In this study we observed that there was a fairly high fecal coliform contamination according to the results of multiple-tube fermentation test (< 1 to 1100 MPN/100 ml).

A total of 23 *E. coli* strains could be recovered from water samples investigated. This is agreed with that previously reported high intensive coliforms and *E. coli* in dug wells water in Lithuania (Malakauskas *et al*., 2007),, in spring and tap water in Turkey (Ozgumus *et al*., 2007). CLOSE *et al*., (2008), detected *E. coli* in all wells, with concentration ranging from < 1 to 2400 MPN/100 ml. Meanwhile, Badrakh *et al*., (2008), reported that 45.4% of all springs had *E. coli* contamination.

Table.1 Sequences and predicted lengths of PCR amplification products of the oligonucleotide primers used

Pathogenic factor	Primers	Primer sequences	Product size (bp)	Reference
Cytotoxic necrotizing factor 1 (*cnf1*)	cnf1a cnf1b	atcttatactggatgggatcatcttgg gcagaacgacgttcttcataagtatc	1105	19 Johnson & Stell (personal communication)
Shigatoxin 1 (*stx1*)	stx1f stx1r	aaatcgccattcgttgactacttct tgccattctggcaactcgcgatgca	366	17 Brian et al., (1992)
Shigatoxin 2 (*stx2*)	stx2f stx2r	cgatcgtcactcactggtttcatca ggatattctccccactctgacacc	282	17 Brian et al., (1992)
Heat labile toxin (*LT*)	lta ltb	tgtttccacttctcttag tattccctgttacgatgt	258	20 Lang et al., (1994)
Heat stable toxin (*ST*)	sta stb	tctgtattatctttcccctc ataacatccagcacacaggc	186	21 Schultz et al., (1994)
Intimin (*eaeA*)	eae1 eae2	tgcggcacaacaggcggcga cggtcgccgcaccaggattc	629	18 Heuvelink et al., (1995)
Cytolethal distending factor (*cdtB*)	cdta 1 cdta 2 cdts1 cdts2	aaatcaccaagaatcatccagtta aaatctcctgcaatcatccagttta gaaagtaaatggaatataaatgtccg gaaaataaatggaacacacatgtccg	430	19 Eric Oswald (Cited from Johnson, and Stell, 2000)
P-Fimbriae (*papA*)	papA-f papA-r	atggcagtggtgtctttggtg cgtcccaccatacgtgctcttc	720	19 Johnson, and Stell, (2000)
S-Fimbriae adhesion (*sfaS*)	sfaS-f sfaS-r	gtggatacgacgattactgtg ccgccagcattccctgtattc	240	19 Johnson, and Stell, (2000)

Table.2 Prevalence of *E. coli* in the water samples collected from different farms of food animals

Animal species	Number of samples	Number of *E. coli* isolates
Camels	9	1
Sheep	18	6
Goats	11	3
Cattle	7	6
Chicken	9	7
Total	54	23

Table.3 Phylogenetic distribution and virulence–related genes of *E. coli* strains recovered from trough water

Phylogenetic groups	No of *E. coli* (n=23)	*chu*A gene	*yia*A gene	TSPEU.C2	Virulence genes encountered (no of isolates
B1	12	-	-	+	stx1, stx2 (4)
A	4	-	+	-	eaeA (1)
B2	4	+	+	+	cnf1, cdtB (2)
D	3	+	-	+	-

Table.4 Antibiogram analysis of *E. coli* isolates recovered from trough water

Antibiotic	No. (%) of resistance (n=23)
Ampicillin (AMP)	7 (30.4)
Amoxycillin- clavulanic acid (AMC)	2 (8.7)
Cefotaxim (CTX)	1 (4.3)
Ceftazidime (CAZ)	0 (0)
Gentamicin (GEN)	0 (0)
Co-trimoxazol (STX)	4 (17.4)
Ciprofloxacin (CIP)	0 (0)
Chloramphincol (CHL)	1 (4.3)
Nalidixic acid (NAL)	1 (4.3)
Streptomycin (STR)	3 (13.04)
Cephalothin (CEF)	2 (8.7)
Tetracycline (TET)	2 (8.7)

Table.5 Antibiotic resistance distribution among *E. coli* recovered from trough water

No. of antibiotics to which isolates were resistant	No. (%) of isolates	Most frequent patterns
0	13 (56.5)	
1	3 (13.04)	AMP
2	3 (13.04)	AMP- CEF, STR-SXT, CHL-NAL
3	2 (8.7)	AMP-CEF-AMC, AMP-SXT-TET
4	1(4.3)	AMP-SXT-TET-STR
5	1(4.3)	AMC-AMP-CTX-STR-SXT
All isolates	23 (100)	

Fig.1 The result of the PCR amplification of the DNA target gene loci of 884-bp fragment DNA region coding for universal stress protein *usp*A.

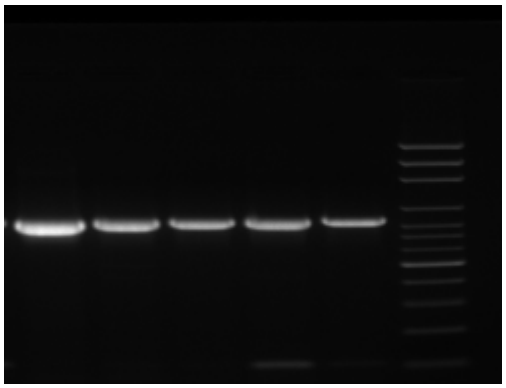
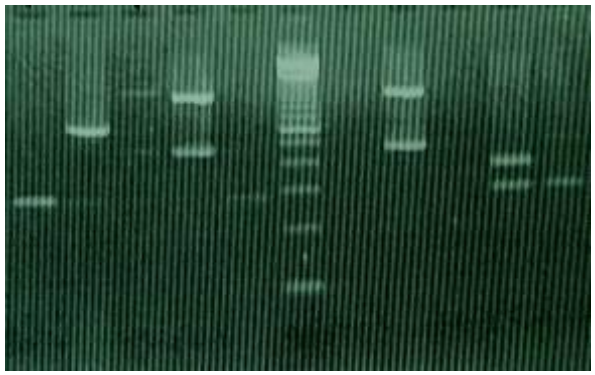


Fig.2 The result of the mPCR amplification of the DNA target virulence-related gene loci of cnf1 (1105-bp), eaeA (629-bp), cdtB (430-bp), stx1 (366-bp), and stx2 (282-bp) fragment DNA regions. Lane (1-10) represent *E. coli* isolates and Lane M, represent 100-bp DNA ladder

1 2 3 4 5 M 6 7 8 9 10



The U.S. environmental protection agency recommends that livestock water contain less than 5,000 coliform organism per 100 ml and fecal coliform should be near zero (Agriculture waste management, 1992).

By the Clermont *et al*., (2000), multiplex PCR procedure, *E. coli* were detected concurrently. In the current study, isolates were analyzed for their phylogenetic background and the *E. coli* strains isolated from the animal drinking water were assigned to group A (4 strains), B1 (12 strains), B2 (4 strains) and D (3 strains). *Escherichia coli* strains can be classified into four phylogenetic groups: A, B1, B2 and D (Herzer *et al*., 1990). The commensal strains are usually placed into the phylogenetic groups; A and B1 and the extraintestinal pathogenic strains into group B2 and, to a lesser extent, group D (Johnson *et al.,* 2001).

Although *E. coli* is a component of normal fecal flora of human and animals, some have the potential of becoming pathogenic and causing opportunistic infections when they acquire virulence genes located on plasmids, bacteriophages or pathogenicity islands (e.g. *stx1*, *stx2* or *eaeA*) (Murray *et al*., 2002). The mPCR of the isolates showed that 30.4% of *E. coli* strains harboring one or more virulence gene markers. In Two studies, one performed with raw surface water and one performed with water from an agricultural waste lagoon, showed that the percentages of *E. coli* isolates possessing virulence genes were 10 and 7%, respectively (Martins *et al*., 1992; Chern *et al*., 2004). In two separate other studies, workers found higher numbers of *E. coli* isolates possessing virulence genes; however, the sampling was done in very different environments. The first study was conducted with water from a German river contaminated with communal sewage, and 41% of the *E. coli* isolates carried virulence markers (Muhldorfer and Hacker, 1994). The other study was carried out with water from a highly polluted South African river and 68% of the *E. coli* isolates were found to possess enteric virulence markers using PCR (Obi *et al*., 2004). Recently, Momba *et al*., (2006), indicated that PCR analysis of the isolates revealed that 75% of the groundwater samples tested positive for *E. coli.*

It is known that water is an important means of spreading STEC between animals and humans. Virulence gene profiling revealed stx_1 and stx_2 gene markers of STEC in four strains. STEC is an etiologic agent of gastrointestinal infections in man and animal. The EaeA, virulence factor correlated with enteropathogenic *E. coli* strains (EPEC) is defined as stx-negative strain able to produce A/E leasions. STEC pathogenicity is mainly attributed to the expression of genes related to the production of cytotoxins (*stx1* and *stx2*) and adherence factors (*eae*) (Barret *et al.,* 1992).

PCR analysis showed that some isolated possessing partial set of virulence hallmarks of extraintestinal pathogenic *E. coli* (ExPEC) (8.7%). These virulence-related genes include toxin (*cnf*1, *cdt*B) genes commonly associated with extraintestinal pathogenic *E. coli* (ExPEC). These isolates may have been commensal isolates which may acquired or lose virulence genes through genetic exchange, either individually or as pathogencity islands (PAIs) in order to better survive in the host. In contrast, other workers found higher numbers of *E. coli* isolates possessing virulence genes; however, the sampling was done in very different environments. Two previous separate studies found high number and diversity of virulence related gens in *E. coli*; all theses isolates were ExPEC;

however, the sampling events and tools used were different. One of these studies was conducted with river water contaminated with communal sewage, and 41% of the *E. coli* isolates carried virulence markers (Muhldorfer and Hacker, 1994), while the other one, was conducted with recreational water and 29% of *E. coli* isolates possessed virulence related genes (Hamelin *et al*., 2006).

Investigations on antibiotic resistance in the aquatic habitat have concerned bacteria of fecal origin because they a used as pollution indicators and may be associated with infectious diseases (Jones *et al.*, 1986). In this study, 10 (43.5%) of *E. coli* isolates showed resistance to one or more antibiotics. Multiresistance was demonstrated in 17.4% of the *E. coli* isolated. The survival potential of fecal coliforms in the environment is related to their ability to acquire antibiotic resistance (Kelch and Lee, 1978). We observed high resistant to ampicillin among the isolates (30.4%). Ampicillin has been found to be very common in *E. coli* isolated from normal microbiota (Toroglu *et al*., 2005; Ozgumus *et al*., 2007). No integrons were detected in the antibiotic resistant *E. coli* isolates. Therefore non integron-related factors, like plasmids may contribute to multidrug resistance as well.

Measures to be taken in order to prevent the spread of STEC. These include stress prevention, controlling food and water quality, as well as feedlot conditions and contact between adult and young animals. The extent of bacterial contamination observed in the drinking water offered to farm animals demonstrates that the animals' daily exposure to *E. coli* from this source alone can be substantial. Multiple factors that influence the survival and persistence of bacteria in natural aquatic systems also appear to have an effect on the complex ecosystems present in animal water trough. This is the first ever on microbial safety of livestock water and *Escherichia coli* harboring virulence-related genes in Saudi Arabia.

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