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Full Length Research Paper

Surface waters in northern Tanzania harbor fecal coliform and antibiotic resistant *Salmonella* spp. capable of horizontal gene transfer

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Some *Salmonella* spp. are important pathogenic bacteria that can be transmitted to people via food and water and that can cause disease characterized by mild to severe enteric and systemic illness. In developing countries, infections caused by pathogenic antibiotic resistant *Salmonella* spp. are a major health challenge, particularly in children. Through the use of membrane filtration and Environmental Protection Agency (EPA) 1200 methods, the presence of fecal coliforms and antibiotic resistant *Salmonella* spp. in surface water sources was investigated, some of which had shared access for animals and people. Out of 100 water samples, 76 were positive for fecal coliforms and 63% of the positive samples contained >100 CFU / 100 ml of water. We observed a significant positive correlation between the number of fecal coliforms and the presence of *Salmonella* spp. (r=0.46, n=100, P=0.01). Importantly, >26% of the samples were positive for *Salmonella* spp. and 88% of these samples harbored isolates resistant to ≥1 antibiotic. Moreover, we found that 26% of antibiotic resistant *Salmonella* spp. isolates were able to transfer their resistance traits to a recipient strain of *Escherichia coli* at the rate of 1-7 x 10^{-3} per donor cells. Microbiological contamination of water was clearly evident in open water sources from northern Tanzania, and the presence of *Salmonella* spp. poses an immediate risk to anyone who consumes these waters if untreated.

Key words: Fecal bacteria, *Salmonella*, invA gene, polymerase chain reaction (PCR), water.

INTRODUCTION

When surface water is contaminated with elevated levels of fecal pollution this can pose a significant health risks for people and livestock that use these waters (Jenkins, 2008). When present *Salmonella* spp. can be transmitted and cause mild to severe enteric and systemic illnesses for example typhoid and paratyphoid (Levantesia et al.,

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2012; Yavari, 2012). Salmonellosis can be self-limiting but it can be severe in children and immunocompromised adults and the elderly (Angulo and Swerdlow, 1995). *Salmonella enterica* serovar Typhi is associated with typhoid fever in humans and is attributed to >600,000 deaths annually worldwide while other serovars do not (Zhang et al., 2003). In developing countries, illness and mortality due to water-borne salmonellosis is increasing (Atherton et al., 1995). For example, of all notifiable diseases in Tanzania, 20% were attributable to typhoid fever alone in 2012 (Mwang'onde et al., 2013).

Antibiotic resistance can exacerbate the Salmonellosis burden (Glynn et al., 1998). Antibiotic resistant bacteria can persistent in different environments due to selection pressure from antibiotic use, and co-selection of genetically linked traits (Call et al., 2008). When resistance confers little fitness cost to the bacteria, resistance can persist while socioeconomic and ecological factors contribute to dissemination of these organisms to other populations (Choffnes et al., 2010). The prevalence of resistant bacteria will be greater where antimicrobial use is more frequent (Goossens et al., 2005), particularly when people can easily access antibiotics without prescription (Plachouras et al., 2010). Commensal enteric bacteria such as some *Escherichia coli* in animals and people probably serve as reservoirs of antibiotic resistance traits (Shehabi and Odeh, 2006). These commensal bacteria have the ability to transfer resistance to other bacteria, including pathogens, through horizontal gene transfer. The ability of bacteria to acquire and disseminate resistance traits via mobile genetic elements such as plasmids has increasingly contributed to the development of multidrug-resistance in the past 50 years (Hawkey and Jones, 2009).

Transmission of coliforms and *Salmonella* spp. primarily occurs via consumption of food and water that is contaminated with fecal pollution (animal and human) (Keddy et al., 2009). *Salmonella* spp. and *E. coli* can survive in water for prolonged periods (>2 weeks) (Haznedaroglu et al., 2012) and can be found in water sources such as rivers, tanks, taps and wells that have been contaminated with human or animal feces (Yavari, 2012). For many years fecal bacteria have been used as indicators of the microbial quality of drinking water (Schriewer et al., 2010). According to World Health Organization guidelines, drinking water supplies should have no detectable *E. coli* per 100 ml samples of water (World Health Organization (WHO), 2011).

In Tanzania *Salmonella* spp, including serovars Paratyphi A, Choleraesuis and Enteritidis, were detected in clinical specimens, milk and water samples (Lubote et al., 2014; Mwang'onde et al., 2013). Importantly, there is a serious lack of potable water in some Tanzanian communities. Most of the polluted surface water resources are shared between animals (livestock and wild) and people on a day-to-day basis. The goal of this study was to ascertain the microbiological quality of select water sources in northern Tanzania and to ascertain the presence of *Salmonella* spp. and the antibiotic resistance phenotypes of recovered isolates.

METHODOLOGY

Sample collection

Water samples were collected from sites that were easily accessed including, ponds, rivers, taps, wells and lake waters from northern Tanzania between March and August, 2014. Samples were collected from Kilimanjaro (Moshi Municipal, Moshi Rural and Hai districts), Arusha (Arusha City, Arumeru, Longido and Monduli Districts), and Manyara (Simanjiro and Babati Districts) regions, after obtaining permission from the local authorities (Table 1). Water samples (100 to 500 ml) were collected in sterile containers and transported in a cooler to the lab and sample processing started within six hours after collection. In this study, we defined rivers as freshwater flowing in defined channels and these waters were typically used for irrigation and livestock watering. Streams were defined as smaller volumes of water that were diverted from the river for irrigation before returning to rivers. We defined tap water as running water supplied by tap (valves) and most of this water originated from springs, upstream river segments or boreholes. Well waters originated form of ground water accessed through drilling or digging and most of the wells in the study area were covered to protect them from contamination. Ponds were defined as open bodies of standing, shallow water created by artificial impoundments designed to capture rain runoff, while lakes were defined as more permanent surface waters that typically have some river or stream input. In the study areas the rainy season falls between March and May months. Rivers were also divided into upstream and downstream segments. Upstream areas experienced little human activity near water sources and were considered at low risk of receiving fecal contamination from human or animal sources. Downstream segments included areas with high risk of impact from fecal contamination and these locations had clear evidence of human and animal interactions.

Detection of fecal bacteria

Bacteria in water samples were detected by using membrane filtration with 47 mm acetate filters with a nominal pore size of 0.45 µm (Cellulose Nitrate filter, Sartorius Stedium Biotech GmbH, Goettingen Germany) (Standard method, 2003). In brief, for each well and tap water sample 100 ml of water was filtered directly. Water samples from rivers, ponds and streams were diluted 1:10 in distilled water before filtering. Sterile distilled water (100 ml) was used as a negative control. All filters were placed on individual M-FC agar plates (M-FC agar, Tulip diagnostics (P) LTD, Goa, India) with rosolic acid (Sigma-Aldrich, St Louis, MO) and incubated aerobically at 44.5±0.5°C for 22- 24 h. Colonies that were light blue in appearance were recorded as fecal coliforms.

Detection of *Salmonella*

Water samples were processed using the EPA Method 1200 with modifications to detect *Salmonella* (EPA, 2012). Briefly, 100 ml bottle water sample was first shaken vigorously and 10 ml of sample (Abakpa et al., 2015) was added to 10 ml of double-strength tryptic soy broth (TSB) (Hi Media Laboratories Pvt. LTD, Mumbai, India) and incubated at 37°C for 24 h to enrich for *Salmonella*. After incubation 30 µl of enriched culture was dropped onto Modified Semisolid Rappaport-Vassiliadis (MSRV, Becton

Table 1. Location of water sampling points in northern Tanzania.

Region	Source	Latitude	Longitude
Arusha	Pond	S03.42581	E036.67314
	River	S03.39126	E036.39126
	Lake	S03.22564	E036.47234
	Tap	S03.36214	E036.68803
	Tap	S03.36837	E036.85860
	River	S03.36829	E036.85867
	Tap	S03.37371	E036.57249
	Pond	S03.37867	E036.55705
	River	S03.32912	E037.15288
	River	S03.37165	E036.79287
	River	S03.37142	E036.81347
	River	S03.37312	E036.82088
	River	S03.37521	E036.83269
	River	S03.36709	S036.83416
	River	S03.36539	S036.83276
	River	S03.36624	S036.83397
	River	S03.36571	E036.83686
	River	S03.37592	E036.84126
	River	S03.37587	E036.84135
	River	S03.50669	E036.87622
	River	S03.36824	E036.89604
	River	S03.55492	E036.96145
	River	S03.44361	E036.85692
	River	S03.43629	E036.85144
	River	S03.43355	E036.85278
	River	S03.43288	E036.85313
	River	S03.39443	E036.81447
	River	S03.39311	E036.82368
	River	S03.39265	E036.82301
	River	S03.38988	E036.83421
	River	S03.39068	E036.83582
	River	S03.39054	E036.83575
	River	S03.38673	E036.83625
	River	S03.43015	E036.85353
	River	S03.37364	E036.57236
	Tap	S03.32912	E036.69996
	Tap	S02.73015	E036.69671
	Pond	S03.37163	E036.57320
	Tap	S02.67736	E036.67984
	Pond	S03.41208	E036.47110
Kilimanjaro	Tap	S03.33246	E037.36866
	Tap	S03.32947	E037.36948
	Tap	S03.32491	E037.33352
	Tap	S03.31773	E037.33358
	Tap	S03.39883	E036.79517
	Tap	S03.34644	E037.34564
	Tap	S03.39739	E036.69127
	Tap	S03.33246	E037.36866
	Tap	S03.33888	E037.33976
	Tap	S03.33383	E037.36945
	Tap	S03.33011	E037.36808
	Tap	S03.34644	E037.34564
	Tap	S03.34162	E037.34837
	River	S03.33059	E037.36587
	Tap	S03.35779	E037.33697
	Tap	S03.28249	E037.30947
	Tap	S03.34094	E037.34882
	Pond	S03.32887	E037.15231
Manyara	Well	S04.19569	E035.74680
	Well	S04.19748	E035.75071
	Well	S04.19762	E035.74953
	Well	S04.24112	E035.74322
	Well	S04.21979	E035.74407
	Pond	S04.21807	E035.75954
	Tap	S04.19666	E035.74781
	Tap	S04.25350	E035.74680
	Tap	S04.23413	E035.74407
	Tap	S03.92235	E035.80795
	Tap	S04.21915	E035.73558
	Tap	S04.19996	E035.74638
	Tap	S04.18349	E035.75299
	Tap	S04.20405	E035.76128
	Lake	S04.23421	E035.74240

Total sampling points 73, some site were sampled twice.

Dickinson and Company Sparks, MD, USA) agar plates with 2% novobiocin. The drops were allowed to dry on the agar plates for 1 h at room temperature and the plates were then incubated at 42°C for 18 h. After incubation, motile *Salmonella* were identified by the formation of pale or whitish halos around the drop. A sterile loop was used to pick the presumptive motile *Salmonella* from the periphery of the halos and these bacteria were then streaked onto xylose lysine desoxycholate (XLD, Hi Media Laboratories Pvt. LTD) plates. The XLD plates were incubated aerobically at 37°C for 24 h. From the XLD plates, distinct colonies having pink to reddish color and a black center were picked and subcultured in Luria-Bertani medium (LB, Difo™ LB Broth Lennox, Sparks, MD USA) broth (one isolate per positive sample). The pure cultures (40 µl) were aliquoted into the wells of a 96-well plate and allowed to completely desiccate in the incubator for 24 h. The 96-well plates were then shipped to Washington State University (Pullman, WA, USA) for phenotypic characterization. Upon reception, the isolates were recovered by adding 150 µl of LB broth to the desiccated cultures followed by overnight incubation at 37°C. After incubation, glycerol (15% final) was added and the plates were stored at -80°C for further analyses that included antibiotic susceptibility profiling, macro-restriction digest assays and conjugation assays.

Assessing antibiotic resistance

Antibiotic susceptibility breakpoint assays (The European Committee on Antimicrobial Susceptibility Testing, 2014) were used to estimate the prevalence of antimicrobial resistant *Salmonella*. For this assay, isolates were considered resistant if they grew on

MacConkey agar plates containing defined concentrations of antibiotics [amoxicillin, 32 µg/ml (Amx, MP Biomedicals, Illkirch, France), ampicillin, 32 µg/ml (Amp, Fisher Scientific, Fair Lawn, New Jersey), chloramphenicol, 32 µg/ml (Chlo, Sigma-Aldrich, St Louis, MO), gentamicin, 16 µg/ml (Gn, LKT Laboratories, Inc., St. Paul, MN), cephalexin, 32 µg/ml (Cn Fisher Scientific), co-trimoxazole, 16/512 µg/ml (Cot, MP Biomedicals), tetracycline, 16 µg/ml, (Tet, GTS, San Diego, CA), and trimethoprim, 8 µg/ml (TRM, MP Biomedicals)]. *E. coli* strain H4H, which was resistant to all tested antibiotics, and *E. coli* strain K-12, which was susceptible to all tested antibiotics were included in each assay as controls for the antibiotic susceptibility test. For quality control, each test was considered valid when there was no growth for *E. coli* K-12 and growth of *E. coli* H4H (Call et al,. 2010) on the agar plates containing antibiotics.

Confirmation of *Salmonella* identity using *invA* PCR

DNA templates for a PCR assay were prepared by centrifuging 2 ml of overnight *Salmonella* cultures at 12,000 g for 10 min. The supernatants were discarded and the pellets were re-suspended in 200 µl of nanopure water and boiled for 10 min in a heat block. The boiled suspensions were centrifuged briefly (5,000 x*g*, 5 min) and 2 µl of the supernatant was used as DNA template for PCR assays. The presence of a diagnostic gene, *invA*, was assessed by PCR (Rahn et al., 1992). A total volume of 15 µl reaction mix (Green PCR master mix, Life Technologies) was used to perform PCR in the thermal cycler (C1000 Touch Thermal Cycler, Bio-Rad, Hercules, CA, USA). The amplified PCR products were visualized using 1.5% agarose gel containing ethidium bromide with a DNA ladder (Gene ruler 1 Kb) as a size standard.

Pulsed-field gel electrophoresis (PFGE)

A total of 26 *inv*A positive *Salmonella* isolates were characterized using Xbal macro-restriction digests with slight modifications of the PulseNet protocol (CDC, 2013). Briefly, overnight culture was adjusted to an optical density of 1.4 using a spectrophotometer (610 nm) and 200 µl of the optical density adjusted culture was augmented with proteinase K (10 µl at 20 mg/ml). Melted SeaKem Gold agarose (FMC BioProduct, Rockville, Maine, USA) was added and gently mixed before dispensing into appropriate plug molds (Bio-Rad, Hercules, CA) for 15 m. Plugs were incubated in ES buffer (0.5 M EDTA, pH 9.0, 1% sodium lauroyl-sacrosine) with proteinase K at 54°C for 1 h and then washed 3X for 1 h in 1 M TE and 0.5 M EDTA buffer. Plugs were then treated with Xbal for 3 h in a water bath at 37°C. Restriction fragments were then resolved by electrophoresis after inserting into 1% SeaKem Gold agarose gels by using a CHEF DRIII apparatus (Bio-Rad). Gels were immersed in 0.5X Tris-borate–EDTA buffer and electrophoresis included initial switching time of 2.2 sec and a final switching time of 68.3 sec, 6 volts and 120° angle. Fragments were resolved at 14°C for 18 h. Control strain *Salmonella enterica* serovar Braenderup (ATCC BAA-664™) was included on every gel to improve accuracy of fragment size estimates. After electrophoresis the gels were stained with ethidium bromide for 20 min and destained 3X for 20 min each with deionized water. Gel images were scanned using ChemiDoc XRS Gel Photo Documentation System (Bio-Rad) and analyzed using BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed using the Unweighted Pair Group Method using arithmetic Averages (UPGMA) with 1% tolerance and a 0.5% optimization setting based on Dice coefficients to quantify similarities.

Conjugation experiments

We used a filter-mating protocol to determine if the AMR traits were transferrable to other bacteria via conjugation. *Salmonella* isolates were mated with a plasmid-free recipient strain (*E. coli* K-12, nalidixic acid resistant, Nal^r) as described previously (Subbiah et al., 2011). Briefly, single colonies of the recipient and donor strains were grown overnight separately in LB broth at 37°C. Equal quantities (10 µl) of overnight each culture were then added on top of a nitrocellulose (~ 1 cm x 1 cm) membrane overlaid onto an LB agar plate with no antibiotics. After 24 h of incubation (37°C), the culture on the membrane was suspended in 500 µl of sterile phosphate-buffered saline (PBS, pH 7.0) and spread onto LB agar plates containing either nalidixic acid (32 µg/ml) and amoxicillin (32 µg/ml) or nalidixic acid (32 µg/ml) and tetracycline (16 µg/ml). Colonies that grew onto these antibiotic containing agar plates were considered transconjugants. The transfer efficiency was calculated by dividing the number of transconjugants by the number of donor cells. Transconjugants were screened for the respective antibiotic resistance phenotypes.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) program version 20 (SPSS Inc., Chicago, IL, USA). Colony counts were analyzed and reported as $\log_{10}$-transformed counts. Analysis of variance (ANOVA) and a post hoc Tukey-Kramer test were used to compare total coliforms counts from different water sources and between different river locations (down and upstream). The correlation between coliform counts and detection of *Salmonella* spp. was estimated by using a nonparametric (Spearman) correlation test.

RESULTS

Out of 100 water samples, 63% had coliform counts >100, 13% had counts between 1 and 100, and 24% were negative for coliforms (Table 2). The mean CFU was significantly higher for pond water samples compared to other water sources (ANOVA, Tukey-Kramer test, $P<0.001$) (Figure 1). CFU counts were significantly higher for the downstream segments compared with upstream segments ($P = 0.01$, Figure 2).

Twenty-six percent of tested water samples were positive for *Salmonella* spp. (isolate identity confirmed by *invA* PCR). All lakes and ponds (n = 9) were positive for *Salmonella* spp. and none of the samples from wells were positive. There was a significant direct correlation between the number of coliforms and the presence of *Salmonella* ($r=0.46$, n=100, $P=0.01$). Twenty-three out of 26 (88.5%) *Salmonella* isolates were resistant to ≥1 antibiotics, with all of these isolates being resistant to amoxicillin (Figure 3). All *Salmonella* isolates were susceptible to ciprofloxacin, gentamicin, cefotaxime and chloramphenicol. Among the resistant isolates 23% (n=6) transferred resistance traits by conjugation with an efficiency that ranged between 1×10^{-3} and 7×10^{-3}. Horizontally transferred resistance included ampicillin, amoxicillin, tetracycline and trimethoprim. Macrorestriction digests assays showed that the 26 *Salmonella* isolates that were tested represented a diverse collection of fragment sizes (Figure 4). The exception was a cluster of five identical PFGE profiles for isolates that were collected from ponds located in the

Table 2. Details of the water samples collected and the detection of fecal coliforms and *Salmonella*.

Regions	Sources (n)[a]	Coliforms range of CFU/ml, n[b]	*Salmonella* spp (number r or s)[c]
Arusha	Lake (1)	BDL[d]	1s
	Pond (7)	>1000, 7	5r
	River (43)	>101-1000,14 >1001, 29	13r, 2s
	Tap (13)	<0, 4 >1-100, 5 >101-1000, 2 >1001, 2	1s
	Well (3)	<0, 3	0
Manyara	Lake (1)	>100-1001, 1	1r
	Pond (1)	>1001, 1	1r
	Tap (8)	<0, 5 >1001, 3	2r
	Well (5)	<0, 3 >1-100, 2	0
Kilimanjaro	Pond (1)	>1001, 1	1r
	Tap (16)	<0, 11 >1-100, 4 >101-100, 1	0
	River (1)	>1001, 1	0

[a]n = number of samples; [b]n = number of samples for the given CFU/ml range; [c]n = number of isolates from each category that were resistant to ≥1 antibiotic (r) or susceptible (s); [d]BDL = below detection limit.

Monduli region.

DISCUSSION

The majority of water samples assessed in this study (76%) exceeded a zero tolerance standard for fecal coliforms in drinking water (WHO, 2011). The average CFU of coliforms was higher in water samples obtained from ponds compared to other water sources. This can be expected given that these ponds are used as water supplies for livestock, wildlife, and people. Ponds of this nature have been identified as sources for transmission of pathogens from cattle to people in Kenya (Jenkins, 2008). During the sampling effort for this project we also observed that rivers and streams were used for bathing, laundering, and some discharge of human waste (Figure 5). Moreover, fecal load was found to be higher in downstream segments compared with upstream segments, which is consistent with anthropological impacts on these water systems. Fortunately, most tap and well water samples did not harbor fecal coliforms.

Also, a statistically significant correlation was detected between the abundance of fecal coliforms and detection of *Salmonella* spp. (r=0.46, n=100, P=0.01). A similar correlation (r <0.32) was documented for surface water in central Florida (Huang et al., 2014). Consequently, coliform monitoring may be predictive for the presence of fecal pathogens such as *Salmonella* in human-impacted waters in Tanzania. This research anticipates that other disease-causing pathogens including *Shigella*, rotavirus, *Campylobacter*, pathogenic *E. coli*, *Cryptosporidium*, and

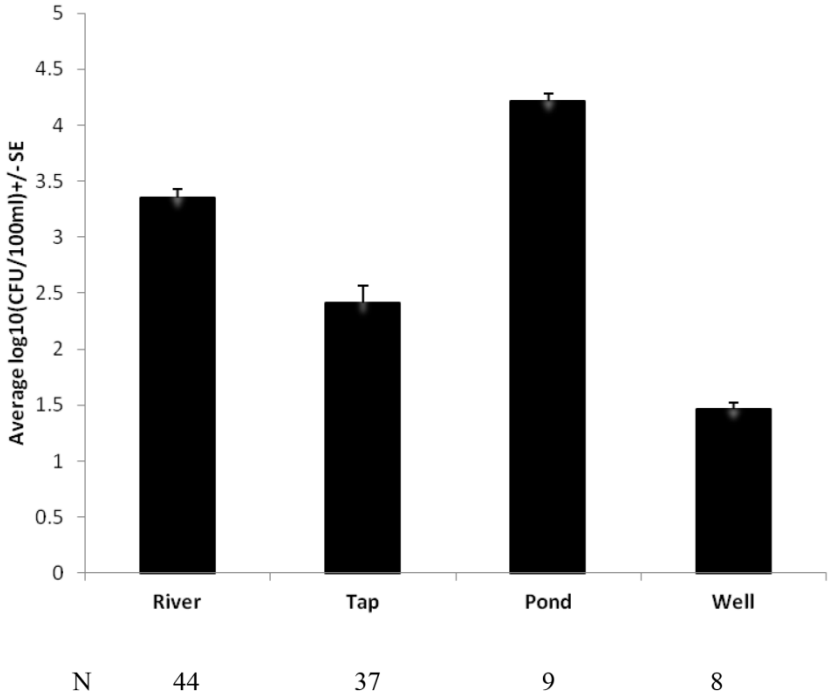

Figure 1. The average number of coliforms detected in various water sources in northern Tanzania. N: Number of samples per source. Lake was excluded from analysis as it has very less sample. Pond harbored significance higher level of fecal bacteria than other sources (ANOVA, Tukey-Kramer test, *P*<0.001).

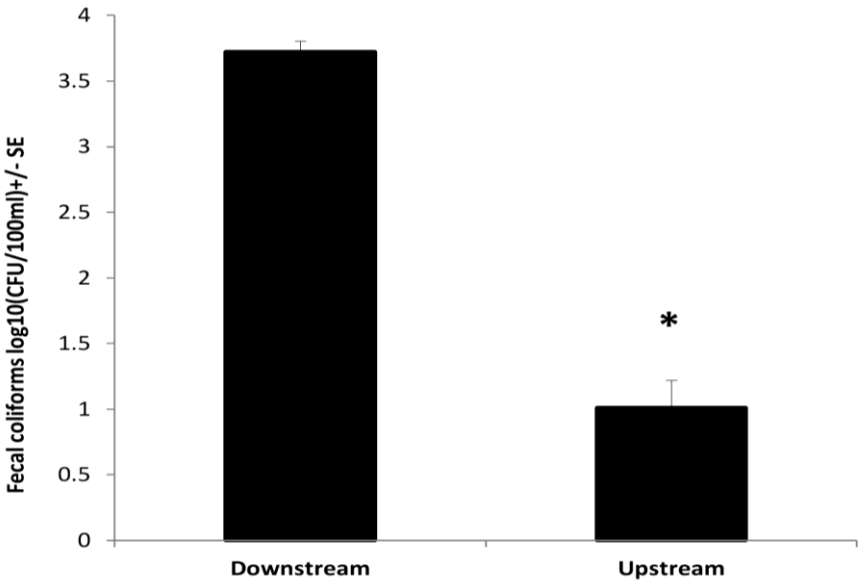

Figure 2. The average number of coliform detected both in downstream and upstream the water sources. N*: Average log10 (CFU/100ml of fecal coliform in upstream. Upstream harbour significance lower cfu compared to downstream segment (*P*=0.01).

Giardia are also associated with these waters. Thomas et al. (2013) sampled waters from urban and rural areas/ agriculture streams in Grand River watershed in Ontario, Canada and found 78.4% were positive for *Salmonella*

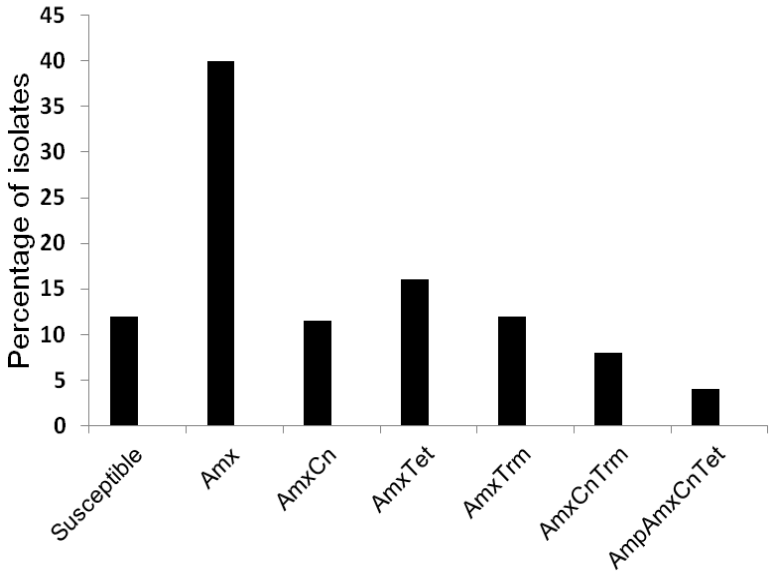

Figure 3. Antibiotic resistance phenotypes for *Salmonella* isolated from different water sources (n = 26). Amp: Ampicillin; Amx: Amoxicillin; Cn: Cefalexin; Trm: Trimethoprim: Tet: Tetracycline. No strains were resistant to ciprofloxacin, gentamicin, cefotaxime or chloramphenicol.

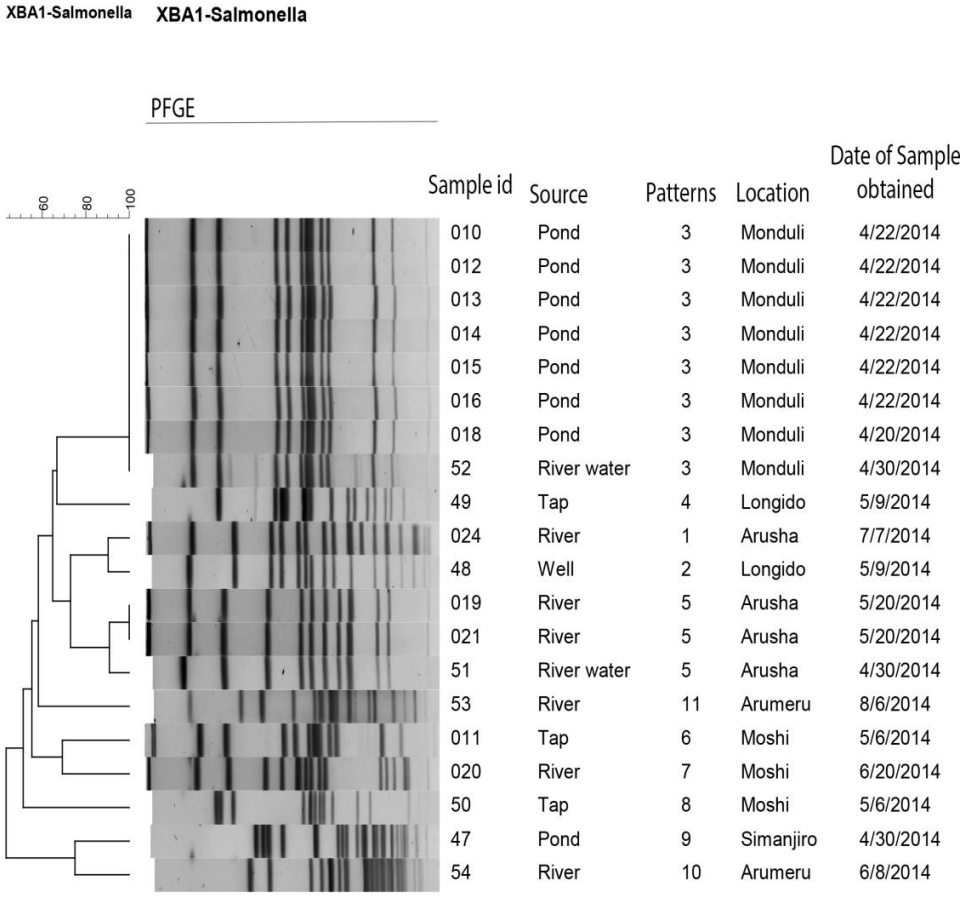

Figure 4. Dendrogram of Xbal PFGE patterns of the *Salmonella* isolates from ponds located near Monduli, Tanzania.

Figure 5. Different human activities along rivers in northern Tanzania that includes laundering.

spp. which is about three times greater than the 26% detected in our study (Thomas et al., 2013). Others have reported lower values including 12.9% from water, soils, and streams in the U.S. (Gorski et al., 2011), and 10.3% from river and irrigation water in Canada (Gannon et al., 2004). Clearly, surface water contamination from human and animal fecal waste is a recurring issue worldwide, and from a public health perspective this is particularly important in areas where people are dependent on these waters for daily needs.

All of the *Salmonella* isolates were motile and positive for *invA*, both attributes consistent with potential to cause disease (Access et al., 2015). The *invA* gene plays an essential step in the invasion of the cells of the intestinal epithelium. Amoxicillin resistance was most common (88% of *Salmonella* isolates) and amoxicillin is considered a first-line antibiotic by WHO to treat UTI infections and pneumonia (Akortha et al., 2011 and PATH, 2014). Antibiotic resistant *Salmonella* spp. were also isolated from different environmental samples including water from Nigeria, where antibiotic resistance was found to all tested antibiotics (Abakpa et al., 2015). Antibiotic-use practices (medical and veterinary) may contribute to this problem in Tanzania (Van Den Boogaard et al., 2009) and efforts to curtail unnecessary antibiotic use are likely to further reduce the overall burden of resistant strains (Carvalho et al., 2013).

In this study resistance traits ampicillin, amoxicillin, tetracycline and trimethoprim were transferred to plasmid-free bacteria via conjugation. Importantly we observed plasmid mediated horizontal gene transfer between *Salmonella* spp. and *E. coli*. Hence is likely that these resistance traits from water isolates can be transmitted back to via direct transmission to people and animals and via indirect transmission through transfer to other bacterial species and pathogens.

The macro-restriction digest assay (PFGE) showed that *Salmonella* isolates from ponds in the Monduli region are clonal. Given that these ponds serve as water supplies for a number of different households and livestock herds, it seems unlikely that they represent a discrete amplification event of a single clone as might be observed during disease outbreaks. It is more likely that the epidemiologic patterns are due to multiple fecal introductions into the environment and/or from a less genetically diverse serovars such as *S. enterica* serovar Enteritidis or Typhi (McEgan et al., 2014). Others have found clonal lineages of *Salmonella* spp. from surface water sources in Florida (McEgan et al., 2014).

If our sampling strategy is representative of other water sources in northern Tanzania, then it is likely that most waterways are polluted with fecal contamination at levels posing health risks to those consuming the water untreated. The presence of fecal coliforms is a moderate predictor for the occurrence of *Salmonella* spp., and consumption of inadequately treated water clearly puts the public at risk of disease in this area. We recommend educating people who use surface water on a daily basis to treat their water (chemical, filtration or boiling) before drinking and to find ways to protect waterways from exposure to animal and human wastes.

Conflict of Interests

The authors have not declared any conflict of interest.

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Antibiotic susceptibility patterns in CTX-M-15-producing *Enterobacteraceae* isolated from healthy Afghan refugees in Iran

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Extended-spectrum β-lactamases (ESBL), including the most wide spread CTX-M-15 enzyme, are major antibiotic resistant mechanisms of *Enterobacteriaceae*. Emergence of this plasmid-mediated enzyme poses a global threat particularly in Asian countries struggling with war. In this study, we investigated CTX-M-15 in clinical isolates of *Enterobacteriaceae* from healthy Afghan refugees in Iran and analyzed the association between CTX-M-15 and pattern of antibiotic resistance as well as the location of this gene among the isolates. No correlation was found between clonal groups and antimicrobial resistance patterns of the *Enterobacteriaceae* species. The frequency of cephalosporin resistance was significantly higher among CTX-M-15-producing isolates compared with other ESBL-producing isolates (P<0.05) with 70 and 30 resistant isolates, respectively; however the association between CTX-M-15 and quinolone resistance was not significant as 4 isolates were quinolone resistant in both CTX-M-15-positive and other ESBL-producing group of isolates. All 20 tetracycline resistant isolates were CTX-M-15-positive (P<0.05) and resistance to aminoglycosides among CTX-M-15-positive isolates were considerably higher (n=22) than other ESBL-producing isolates (n=7) (P<0.05). Resistance to meropenem, imipenem, aztreonam, piperacillin and ampicillin was not significantly associated with CTX-M-15-production. Plasmid analysis revealed that the CTX-M-15 gene is located on a large plasmid ranged between 90 and 100 kb. This is amongst the premier report describing the association between CTX-M-15-production and different antibiotic resistance patterns in *Enterobacteriaceae* isolates collected from healthy individuals. The significant association between cephalosporin, aminoglycoside and tetracycline resistance and CTX-M-15-production emphasizes a need for introducing new antibiotic choices for the treatment of infections caused by *Enterobacteriaceae*.

Key words: Extended-spectrum β-lactamases (ESBL), CTX-M-15, *Enterobacteriaceae*, antibiotic resistance, plasmid.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) represent a major threat among *Enterobacteriaceae* species (Jemima and Verghese, 2008; Leflon-Guibout et al., 2004). ESBLs are capable of hydrolyzing β-lactam antibiotics, and are

plasmid-mediated β-lactamases that are easily transferable among different bacteria (Nemec et al., 2004). The production of ESBLs in *Enterobacteriaceae* confers resistance to the most of cephalosporins that have been commonly used to treat gram negative bacterial infections (Poirel et al., 2002). Combination therapy with β-lactams and aminoglycosides is one of the alternative choices for the treatment of systemic infections caused by *Enterobacteriaceae* (Livermore, 1995). However, co-resistance to non-β-lactam antibiotics such as aminoglycosides is also frequent, by the co-location and thus co-transfer of the resistance determinants in the same genetic elements. The β-lactamases can also be associated with quinolone resistance (Pitout and Laupland, 2008). The main mechanism of quinolone resistance was previously attributed to chromosomal mutations; however, since 1998, various plasmid-mediated horizontally transferable quinolone resistance genes have been reported worldwide in clinical isolates of *Enterobacteriaceae* (Jemima and Verghese, 2008; Pitout and Laupland, 2008; Mac et al., 2010; Sidjabat and Paterson, 2015). The emergence of concomitant quinolone resistance in ESBL-producing *Enterobacteriaceae* is a public health issue because the inappropriate use of quinolones will not only promote quinolone resistance, but will also raise transfer of ESBL genes on the same plasmid as the plasmid-mediated quinolone resistance genes. Thus, the recent worldwide increase in the ESBL-producing *Enterobacteriaceae* has resulted in a therapeutic dilemma, as the antibiotic choices are limited because of ESBL production.

In recent decades, the ESBLs of the TEM, SHV and CTX-M type have emerged as significant mechanisms of resistance in Gram-negative bacilli including *Enterobacteriaceae* species (Apisarnthanarak et al., 2008); however, the most widespread plasmid-mediated ESBLs nowadays are the CTX-M enzymes (Hernandez et al., 2011). The first CTX-M-type β-lactamases were identified as plasmid-encoded enzymes in clinical isolates from the *Enterobacteriaceae* (Bogaerts et al., 2007). Five different groups of CTX-Ms containing a total of over 100 different types, have been described so far, among which, CTX-M-15 type which belongs to group 1 CTX-M enzymes, is the most widespread in *Enterobacteriaceae* strains (Jemima and Verghese, 2008).

Since the mid-1990s, CTX-M-positive strains have been identified in most parts of the world including Asia, Europe, North America and South America (Bogaerts et al., 2007). However, little is known about the CTX-positive strains available in the Middle East, particularly among individuals from war-torn countries such as Afghanistan, which can result in the emergence of multi-drug resistance (MDR) species that can spread even worldwide over time due to lack of sufficient health care supports in these countries.

In this study, we investigated the existence prevalence of CTX-M-15 gene in *Enterobacteriaceae* isolates from healthy Afghan refugees in Iran and analyzed the antibiotic susceptibility patterns in CTX-M-15-positive strains.

MATERIALS AND METHODS

Clinical samples and bacterial isolates

We conducted a prospective review of the electronic and manual medical records of nearly 2500 newly arrived refugees at 100 specialized primary care clinics for Afghan refugees in Iran between August 2010 to February 2013. Newly arrived refugees were defined as persons, other than travelers or tourists, who had arrived in the last one month (less than 30 days) to Iran from their usual residence; Afghanistan, living in camps specialized for Afghan refugees with the same living conditions. Data were drawn from the earliest available standardized screening test results recorded in refugees' charts as part of routine care for the newly arrived refugees. 1112 refugees who were without any urinary tract infection (UTI) and enteric infection sign and symptoms, as well as any underlying illness and no previous antibiotic treatment during the last 6 months, were subjected to our study in order to investigate *Enterobacteriaceae* healthy carriers. 678 fecal and 434 urine (total: 1112) specimens were collected in the camps, delivered in sterile plates and processed within 2 h after sampling. At least one sample (fecal and/or urine) was taken from a single refugee. MacConkey agar and Eosin Methylene Blue (EMB) agar designed to selectively isolate Gram-negative and enteric bacteria were inoculated, incubated aerobically at 37°C and examined after 24 and 48 h. *Enterobacteriaceae* species were identified by the standard microbiological methods and biochemical tests (Shahid et al., 2011).

Antimicrobial susceptibility testing and confirmation of ESBL production

Antimicrobial susceptibility testing was performed on Muller Hinton agar (Trek Diagnostic System Ltd., West Sussex, United Kingdom) by the standard disk diffusion method as per Clinical Laboratory Standard Institution (CLSI) guidelines (Boyd et al., 2004). *K. pneumoniae* ATCC700603 was used as control. Additionally, the MICs of the antibiotics were determined by broth microdilution method according to the Clinical Laboratory Standard Institution (CLSI) guidelines, as well. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organisms. The antibiotic disks (Mask, UK) used and their MICs are shown in Table 1.

ESBL detection was done by a disk diffusion method. The test inoculum (0.5 McFarland turbidity) was streaked on Muller Hinton agar. A disk of ceftazidime-clavulanic acid (30/10 µg/ml) was placed at a distance of 30 mm, center to center, from ceftazidime (30 µg/ml). A parallel experiment was carried out using cefotaxime-clavulanic acid (30/10 µg/ml) and cefotaxime (30 µg/ml) to ensure ESBL production. A ≥ 5 mm increase of the zone diameter for the clavulanic-supplemented disks compared with the zone diameter

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Table 1. MICs of CTX-M-15-positive and other ESBL-producing *E. coli* and *K. pnemoniae* isolates to various antibiotics tested

Antibiotics Tested	MIC(µg/ml)				Resistant	Susceptible
	K. pneumoniae		*E. coli*			
	CTX positive	Other ESBL-producing isolates	CTX positive	Other ESBL-producing isolates		
Meropenem	≤0.5	≤0.5	≤0.5	≤0.5	1	99
Aztreonam	≥64	16	≥64	8	13	87
Gentamicin	≥16	8-16	≥16	8	16	84
Ciprofloxacin	≥32	8	4	2	8	92
Amikacin	32	8	8	≤8	11	87
Imipenem	0.25	<0.25	0.25	<0.25	1	99
Cefotaxime	≥128	64	≥128	≤4	18	82
Cefepime	≥64	32	16	4	12	88
Tetracycline	4	2	4	2	20	80
Ampicillin	>64	64	>64	64	40	60
Piperacillin	>512	16	>512	4-16	10	90
Ceftriaxone	>256	4	>128	4	20	80
Cefpodoxime	≥128	64	64-128	32	26	74
Ceftazidime	≥128	≤2	32	≤2	24	76

Table 2. Sequences of primers used for CTX-M-15 gene detection.

Target gene	Primer	Sequence (5`-3`)
$bla_{CTX\text{-}M\text{-}15}$	CTX-M-15/28F	ATAAAACCGGCAGCGGTG
	CTX-M-15/28R	GAATTTTGACGATCGGGG

for the plain disks was considered to indicate the presence of ESBL.

Random amplified polymorphic DNA (RAPD) typing

In order to distinguish whether ESBL-positive isolates were identical or different clones, a RAPD PCR typing assay was performed. Two different primers were used for RAPD; 5`- CCGCAGCCAA- 3` for *Escherichia coli* and 5`- CGTGGGGCCT- 3` for *Klebsiella pneumoniae*. Total bacterial DNA was extracted using AccuPrepTM Genomic DNA Extraction Kit (Bioneer, Daejon, South Korea). RAPD polymerase chain reaction (PCR) was performed in a 20 µl AccuPowerTM PCR PreMix (BioNeer) with 100 pmol of each primer as follows: initial denaturation at 92°C for 2 min, followed by 40 cycles of 92°C for 30 s, 40°C for 1 min and 72°C for 1.5 min, and a final incubation at 72°C for 10 min.

The experiments were repeated twice to assess reproducibility. The amplified DNA fragments were separated on 2% (w/v) agarose gels, stained with ethidium bromide and photographed under ultraviolet light. DNA fingerprints were compared by visual inspection. Similar isolates with the same banding pattern were assigned to the same RAPD type.

Plasmid analysis

For the analysis of plasmids, plasmid DNA was obtained from the isolates using QIAGEN plasmid Midi Kit (Qiagen, Hilden, Germany), following the manufacturer`s instructions to screen for plasmid-encoded CTX-M-15 gene. Plasmids were digested with *EcoRI* or *BamHI*, and the resulting restriction fragments were separated by electrophoresis in a 1% agarose gel.

Detection of CTX-M-15 by PCR

The PCR was done on the bacterial whole genome to detect the CTX-M-15 gene. The primers used for the detection of CTX-M-15 gene are shown in Table 2. Briefly, bacterial DNA was prepared by suspending one loop of fresh colonies in 500 µl of sterile distilled water and heating the mixture at 95°C for 10 min. The reaction was carried out in a total volume of 50 µl. The cycling conditions were: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s; 56°C for 30 s and 72°C for 1 min; and a final elongation at 72°C for 10 min.

Sequencing of representative isolates

PCR products were sequenced by BioNeer Co., South Korea and results were investigated using MEGA-4 and Chromas 1.45 softwares. The nucleotide sequences results thus obtained were compared with sequences from Genbank nucleotide database at www.ncbi.nlm.nih.gov/blast.

Statistical analysis

The significance of association between CTX-M-15 gene and

Table 3. Antimicrobial susceptibility testing results for CTX-M-15-positive and other ESBL-producing *E. coli* and *K. pneumoniae* isolates

Antibiotic Tested	*K. pneumoniae*				*E. coli*			
	CTX-M-15 producers (n=8)		Other ESBL producers (n=30)		CTX-M-15 producers (n=24)		Other ESBL producers (n=38)	
	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
Meropenem	1	7	0	30	0	24	0	38
Aztreonam	5	3	3	27	5	19	0	38
Gentamicin	7	1	3	27	5	19	1	37
Ciprofloxacin	2	6	3	27	2	22	1	37
Amikacin	6	2	1	28	4	20	0	37
Imipenem	1	7	0	30	0	24	0	38
Cefotaxime	8	0	1	29	8	16	1	37
Cefepime	4	4	2	28	4	20	2	36
Tetracycline	8	0	0	30	12	12	0	38
Ampicillin	5	3	14	16	8	16	13	25
Piperacillin	6	2	0	30	4	20	0	38
Ceftriaxone	8	0	2	28	8	16	2	36
Cefpodoxime	7	1	5	25	10	14	4	34
Ceftazidime	7	1	10	20	6	18	1	37

antibiotic resistance was analyzed by Fisher test and a P value of <0.05 was considered significant.

RESULTS

Clinical specimens and bacterial isolates

Of 1112 fecal and urine specimens, obtained from 637 male (mean age: 31±3) and 475 female (mean age: 29±2) refugees, 138 samples (96 fecal and 42 urine) yielded growth of Gram-negative bacteria from which, 130 were identified as *Enterobacteriaceae* species (82 *E. coli* and 48 *K. pneumoniae*) using microbiological methods and biochemical tests. ESBLs were phenotypically detected in 100 isolates (9% of the total 1112 samples) using ceftazidime as well as cefotaxime as an ESBL inhibitor including 62 *E. coli* and 38 *K. pneumoniae*.

RAPD analysis

RAPD assay for 62 ESBL-positive *E. coli* isolates revealed 3 clonal groups representing ≥ 2 isolates with > 80% similarity. These three clonal groups represented 58 of the 62 isolates; 4 isolates represented a single unique pattern.

RAPD assay for 38 ESBL-positive *K. pneumoniae* isolates also revealed 2 clonal groups representing ≥ 2 isolates with > 80% similarity. These 2 clonal groups represented 36 of the 38 isolates; 2 isolates represented a single unique pattern.

Antibiotic susceptibility testing results

The antimicrobial susceptibility testing results for the 100 ESBL-producing isolates and the association between CTX-M-15-production and antibiotic resistance pattern is shown in Table 3. *E. coli* and *K. pneumoniae* clones did not differ significantly with respect to the number of antimicrobial resistance patterns (P= not significant). Among the 100 ESBL-producing isolates, 99 were susceptible to meropenem and only 1 CTX-M-15-positive, was found to be resistant (P= not significant). The same pattern was seen for imipenem. 10 CTX-M-15-positive and 3 other ESBL-producing isolates were resistant to aztreonam (P= not significant) whereas all 10 resistant isolates to piperacillin were CTX-M-15-positive (P= not significant). The frequency of cephalosporin resistance, especially for cefpodoxim with 26 resistant isolates, was significantly higher among CTX-M-15-producing isolates compared with the other ESBL-producing isolates (P<0.05); however, among both CTX-M-15 (n=4) and other ESBL-producing isolates (n=4), 8 were resistant to quinolone (P=not significant). High resistance to ampicillin was detected among CTX-M-15 (n=13) and other ESBL-producing isolates (n=27) (P=not significant) while resistance to aminoglycosides among CTX-M-15-positive isolates were considerably higher (n=22) than other ESBL-producing isolates (n=5) (P<0.05). All 20 tetracycline resistant isolates were CTX-M-15-positive (P<0.05).

Plasmid analysis and CTX-M-15 gene location

32 isolates (including 24 strains of *E. coli* and 8 strains of

K. pneumoniae) of the 100 ESBL-producers confirmed phenotypically, had CTX-M-15 gene detected by PCR. PCR revealed that the CTX-M-15 gene was located on a large plasmid. This large plasmid carried CTX-M-15 gene confirmed by gene sequencing of the representative isolates (accession number: KF723592.1 for *E. coli* and KF513160 for *K. pneumoniae*). The size of this large plasmid harboring CTX-M-15 gene determined by the analysis of the *EcoRI* and *BamHI* restriction enzymes were estimated to range between 90 kb and 100 kb for all CTX-M-15-positive isolates, as previously investigated from Bosnia and Herzegovina and India (Poirel et al., 2002).

DISCUSSION

The CTX-M family of ESBLs has been increasingly detected worldwide (Ma et al., 2009). Dominant emergence of CTX-M types has been observed in Asia (Sidjabat and Paterson, 2015; Eckert et al., 2004; Robicsek et al., 2006; Stiles et al., 1981); recently high prevalence of CTX-M-15 was reported from South India as an Asian country neighboring Middle East countries (Ensor et al., 2006). However, data on CTX-M epidemiology in war-torn Asian countries such as Afghanistan are scarce. In the present investigation, our data suggest that CTX-M-15 could be one of the most prevalent genes among ESBL-producing *Enterobacteriaceae* isolates collected from Afghan refugees in Iran according to the fact that CTX-M-15 was present in about one third of the 100 ESBL-producing isolates confirmed by DNA sequencing.

In this study, only 32% of the isolates demonstrated the presence of CTX-M-15 gene as opposed to that of 100 phenotypically detected ESBL-producing isolates and we speculate that some other ESBL genes are also prevalent in the Middle East bacteria which we did not look for in this study. The antimicrobial resistance of gram-negative organisms has built up progressively during the last few decades, leading to increased incidence of outbreaks of infections due to existence of multi-resistant bacteria (Poirel et al., 2002). This issue is of great concern in war-torn countries since disrupted health care systems in these countries may result in rapid spreading of the resistant bacterial strains and thus incidence of outbreaks of infections. We also demonstrate that CTX-M-15 gene was found to be encoded on large transferable plasmid that might contribute to the endemic outbreak of infections that may be faced in countries struggling with war.

Clinical isolates expressing CTX-M β-lactamases often display high resistance to cephalosporins. In this collection of isolates, 34 and 36 of the CTX-M-15-producing *K. pneumoniae* and *E. coli* were resistant to cephalosporins, respectively. CTX-M-15 belongs to the first group of the CTX-M β-lactamases with measurable ceftazidime hydrolysis that results in higher ceftazidime resistance (Soge et al., 2006); however, in our study resistance to cefpodoxim was higher among CTX-M-15-positive isolates. Thus, universal susceptibility to cephalosporins in *Enterobacteriaceae* is no longer guaranteed as a result of high resistance rates to different members of this group of antibiotics since decades (Nemec et al., 2004).

Molecular characterization of plasmids encoding CTX-M-15 from gram-negative strains involved in outbreaks in different countries has demonstrated that they additionally carried other antibiotic resistance genes (Bonnet, 2004; Kanamori et al., 2011; Karisik et al., 2006). Our results show that 22 of the CTX-M-15-producing isolates were resistant to aminoglycosides which is similar to reports from other studies (Mac et al., 2010; Apisarnthanarak et al., 2008; Lavollay et al., 2006; Strahilevitz et al., 2009).

There is a possibility that *Enterobacteriaceae* harboring CTX-M-15 gene have reduced susceptibility to quinolones than other ESBL-producing strains (Pitout and Laupland, 2008; Shahid et al., 2011), whereas in this study significant quinolone resistance among CTX-M-15-positive isolates was not seen compared with other ESBL-positive isolates.

We revealed in the present study that the frequency of tetracycline resistance among CTX-M-15-producing isolates was considerably higher as all tetracycline resistant isolates had CTX-M-15 gene; this finding is consistent with the hypothesis that CTX-M genes are associated with large plasmids that also carry tetracycline resistance genes (Hernandez et al., 2011; Dedeic-Ljubovic et al., 2010; Lee et al., 2006).

We found no correlation between clonal groups and antimicrobial resistance patterns since quinolone susceptibility and tetracycline resistance was seen in all *E. coli* and *K. pneumoniae* clonal groups independently. This indicates that antimicrobial resistance patterns cannot be predicted from the clonal status of the *Enterobacteriaceae* species.

In conclusion, we identified CTX-M-15 in ESBL-producing *Enterobacteriaceae* from the Afghan refugees, and also found a significant association between CTX-M-15 and cephalosporin, aminoglycoside and tetracycline resistance. Our data emphasize the importance of detecting antimicrobial resistance genes such as CTX-M group particularly in Asian countries with disrupted health care systems due to war in order to promote appropriate antimicrobial therapy and effective infection control. Future regional epidemiological data on antimicrobial resistance throughout Asia, including the war-torn countries such as Afghanistan, Iraq and Syria, will be required to implement strict national antibiotics policies to restrict the spread of these resistance bugs.

Conflict of interests

No competing financial or personal interests exist in any

part of the study.

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Ethical considerations

Study design and study materials were proposed to committee of ethics of the corresponding university. All individuals were described in the study design, study methods and were all aware of risks and benefits of study. All individuals signed informed consent form. Ethical committee of medical research at “Shahid Beheshti University of medical sciences” approved the protocol.

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Potential of bamboo vinegar with liquid probiotics on growth performance, fecal microbiology and fecal odorous gas emissions from finishing pigs

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In this study, the effects of dietary supplementation with bamboo vinegar liquid probiotics (BVLP) on finishing pigs were investigated. A total of 105 crossbreed (Landrace × Yorkshire) finishing pigs were allotted to five treatments of three replicate pens (7 pigs/pen) following a completely randomized design. Results elucidated that, there were no significant differences observed in weight gain, feed intake and feed conversion efficiency among the dietary supplementation. Carcass weight and carcass yield grade did not differ significantly; however, the back fat thickness tended to be decreased in the 0.2% BVLP group compared to control (P<0.10). In addition to that, fecal *Escherichia coli* content was significantly suppressed in response to the antibiotic and 0.2, 0.4, and 0.8% BVLP supplementation relative to the control (P>0.05); where no significant differences were found between antibiotics and BVLP supplemented groups. Furthermore, among malodorous gases, ammonia and hydrogen sulfide gas was significantly down trended in the antibiotic and 0.2, 0.4, and 0.8% BVLP supplemented groups and no significant differences were observed between antibiotic and BVLP supplemented groups. Significant diminution of the sulfur dioxide was found after antibiotic and 0.2, 0.4, and 0.8% BVLP supplementation compared to control (P<0.05); where antibiotic and 0.4% BVLP also significantly differed. Moreover, the mercaptan gas was substantially decreased in the 0.4% BVLP supplemented group relative to control (P<0.05), while no significant differences were observed between the antibiotic and BVLP groups. Taken together, supplementation of BVLP could be potentially utilized in the diet of finishing pigs for the suppression of the fecal pathogenic *Escherichia coli* and fecal odorous gas emissions, without negative impact on the growth performance.

Key words: Bamboo vinegar liquid probiotics, gaseous emissions, microbiology, growth performance, finishing pigs.

INTRODUCTION

Animal production is increasing rapidly in Asia (Huynh et al., 2007). While developing countries produced 31% of the world's meat in 1980, they are expected to produce 60% by 2020 (Delgado et al., 1999). Among the projected 60%, around 13.2% will be produced in Southeast Asia alone. In such countries, pigs serve as an important source of family income (Steinfeld, 1998). Feed additives are compounds added to diets to enhance animal performance, either directly or indirectly. Antibiotics, which are the most controversial additive, were used at

the therapeutic level for disease treatment (high dose-short duration) or at the sub-therapeutic level for growth promotion (low dose-long duration) until last decades. A great deal of debate regarding the continued use of sub-therapeutic antibiotics in livestock feeds has taken place due to concern surrounding the development of antibiotic resistant bacteria. In fact, the European Union has banned the use of growth promoters in the feed of food animals (European Commission, 1998), and similar efforts are being made in Denmark, Korea, the United States and elsewhere throughout the world. Therefore, pork producers are looking for replacements for growth-promoting antibiotics.

Among various antibiotic alternatives, use of probiotics is a common approach that has shown potential. Probiotics are a class of feed additives composed of living bacteria and/or yeast cultures that are provided to improve desirable microflora balance within the small and large intestine (McKean, 2004). Most common mixtures contain one or more of the *Lactobacillus* species, *Bacillus subtilis, Streptococcus faecium*, *Saccharomyces cerevisiae* and other commensal species. These mixtures are thought to work either directly, by excluding harmful bacteria or reducing intestinal pH, or indirectly, by favoring the development of other desirable health promoting microorganisms that compete with harmful bacteria to reduce their presence in the gut. The desired effect is improved weight gain and feed efficiency via improved gut digestion and reduced pathogenic organism loads. Ko and Yang (2008) found that finishing pigs receiving probiotics in their feed showed equal or superior daily gain, intake, and feed efficiency as pigs fed antibiotics.

Various active substances are present in medicinal plants or extracts, including natural antibiotics. Accordingly, functional medicinal plants have been applied as replacements for antibiotics (Harris and Webb, 1990; Martin and Nisbet, 1992; Berg, 1998; Lyons and Jacques, 2000; Kwon et al., 2005; Sarker et al., 2010d). Among medicinal plants, bamboo and bamboo vinegar are very effective due to their natural active substances. Bamboo vinegar is a by-product of bamboo carbonization derived from the smoke of carbonized bamboo that has a unique tart flavor and smoky smell. Bamboo vinegar contains over 200 ingredients that are all from natural sources and known to be safe for use on humans and animal. The acetic acid contained in bamboo vinegar softens the skin cuticle and relieves dermatitis, itch, and athlete's foot. The functional and medicinal properties of bamboo vinegar, such as its antioxidant, antimicrobial, and antimutagenic activities have been studied for decades (Lin et al., 2008), and it has recently drawn the attention of medical researchers. Suga et al. (2003) isolated an antioxidative phyllostadimier from *Phyllostachys edulis*, a common bamboo species. The combination of medicinal plants with probiotics has shown the potential to improve many aspects of broilers (Sarker et al., 2010a, 2010b, 2010c), pigs (Ko and Yang, 2008) and calves (Sarker et al., 2010e). To the best of our knowledge, there were limited researches has been conducted on the combination of bamboo vinegar and probiotics on the growth performance, fecal microbiology and fecal odorous gas emissions in finishing pigs. Odorous gas emissions from the swine industry are considered as a major problem and continuous research has been conducted to minimize the emissions through different dietary manipulations (Van der Peet-Schwering et al., 1999). This study was conducted to investigate the effects of bamboo vinegar liquid probiotics (BVLP) on fecal microbiology and fecal malodorous gas emissions from finishing pigs and to ascertain whether it has a positive or negative impact on the growth performance of the finishing pigs.

MATERIALS AND METHODS

Animal and experimental design

A total of 105 crossbreed (Landrace × Yorkshire) finishing pigs were housed in concrete floor pens for a period of six weeks. The pigs were assigned to five dietary treatments in a completely randomized design. Each treatment consisted of three replicates with seven pigs per replication. The five dietary treatments were a control (no additives), antibiotics (control diet + 30 ppm chlortetracycline), and diets supplemented with 0.2, 0.4 or 0.8% BVLP. The nutrient composition of the control diet (Table 1) was in accordance with the suggested nutrient requirements for finishing pigs (NRC, 1994). BVLP used in this experiment were composed of 17.73% crude protein, 2.90% crude fat, 9.71% crude fiber and 10.37% crude ash. In addition, 4.2×10^7 cfu/g *Lactobacillus acidophilus*, 5.8×10^6 cfu/g *Lactobacillus plantarum*, 2.6×10^7 cfu/g *B. subtilis* and 6.2×10^9 cfu/g *S. cerevisiae* were included in the BVLP (Table 2). The chemical compositions of the five experimental feeds were analyzed as described by the AOAC (1990) (Table 3). The experimental protocol was applied based on the guidelines of the Animal Care and Management Committee of the Sunchon National University, Republic of Korea.

Measurements and analyses

Body weight, feed intake, and feed conversion ratios were measured every two weeks. Analyses of carcass grade, fecal microbiology, and malodorous gas emissions were conducted at the end of the experiment as described subsequently.

Measurement of body weight, feed intake and feed conversion ratio

The body weight of pigs was measured every two weeks from the

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Table 1. Formula and chemical composition of basal diet for finishing pigs.

Ingredient	Amount (%)
Yellow Corn	45.15
Wheat	25.00
Wheat bran	4.00
Soybean meal	16.00
Limestone	0.78
Calcium phosphate	1.10
Salt	0.25
Vit-min. premix[1)]	0.55
Animal fat	2.50
Molasses	4.50
L-Lysine	0.17
Chemical composition[2)]	
ME (kcal/kg)	3,265.00
Crude Protein (%)	16.00
Ca (%)	0.50
Available P (%)	0.45
Lysine (%)	0.80
Methionine (%)	0.27

[1)]Vit-min. mix provided the following nutrients per kg of premix: vitamin A, 6,000 IU; vitamin D_3, 800 IU; vitamin E, 20 IU; vitamin K_3, 2 mg; thiamin, 2 mg; riboflavin, 4 mg; vitamin B_6, 2 mg; vitamin B_{12}, 1 mg; pantothenic acid, 11 mg; niacin, 10 mg; biotin, 0.02 mg; Cu (copper sulfate), 21 mg; Fe (ferrous sulfate), 100 mg; Zn (zinc sulfate), 60 mg; Mn (manganese sulfate), 90 mg; I (calcium iodate), 1.0 mg; Co (cobalt nitrate), 0.3 mg; Se (sodium selenite), 0.3 mg[2] Calculated value.

initial day to the final day of the experiment to calculate body weight gain. The feed intake of pigs was recorded every two weeks by offering a weighed quantity of feed and weighing the residue. The feed conversion ratio was calculated by dividing feed intake by the body weight gain of pigs.

Measurement of meat carcass grade

Carcass quality traits in terms of slaughter weight, back fat thickness, and carcass grade were determined according to the Korean carcass grading system (Animal Products Grading Service, 1998).

Microbial analysis

For *Escherichia coli* and *Salmonella* enumeration, fecal sample of two pigs from each treatments were collected at the end of the experiment. Fresh fecal samples were collected from the rectum of the pigs in sterile polythelene bags. During fecal collection stimulation of internal and external sphincters were applied to avoid contamination of the samples. In brief, 1 g aliquots of feces from each treatment group were diluted in 10 ml of saline solution, after which 1 ml was serially diluted to make dilutions of 10^1 to 10^{11}. Microbial plates were then inoculated with three dilutions of each. Samples were plated on MacConkey Sorbitol Agar and Salmonella Shigella Agar for *E. coli* and *Salmonella*, respectively. Triplicate agar plates were then incubated at 37°C for 24 h. The total number of colonies for each *E. coli* and *Salmonella* were enumerated. Finally, the bacterial counts were expressed as $\log_{10}$ cfu/ml.

Table 2. The name, strain and number of microflora used for fermentation of bamboo vinegar liquid with chemical composition.

Item	Contents
Number of microflora in BVLP [1)]	
Lactobacillus acidophilus KCTC 3111	4.2×10^7 cfu/g
Lactobacillus plantarum KCTC 3104	5.8×10^6 cfu/g
Bacillus subtilis, KCTC 3239	2.6×10^7 cfu/g
Saccharomyces cerevisiae KCTC 7915	6.2×10^9 cfu/g
Chemical composition (g/100g) [2)]	
Moisture	8.98
Crude protein	17.73
Crude fat	2.90
Crude fiber	9.71
Crude ash	10.37

[1)]BVLP=bamboo vinegar liquid probiotics. [2)]Calculated chemical composition.

Gas measurements

At the end of the experiment, emissions of malodorous gases from feces were measured using a handheld GASTEC-GV 100 cylinder pump (Gastec Corporation, Japan). Specifically, ammonia (NH_3), hydrogen sulfide (H_2S), sulfur dioxide (SO_2), and total mercaptans (CH_3SH) were measured using individual gas detector tubes. Gastec detector tube (3 LA, 3M for NH3; 4 LB, 4LK for H_2S; 5 LA for SO_2; 70L for Mercaptan). During measurement, the tube was open and 100 ml of headspace air was sampled approximately 2.0 cm above the sample surface. The concentration of lethal gases was expressed as ppm/100 ml.

Statistical analysis

The data obtained were analyzed using the SAS Package Program (1995) to estimate variance components for a completely randomized design. Duncan's multiple range tests (1955) were used to identify significant differences between treatment means. A $P<0.05$ was considered to indicate significance; while a tendency was considered at $P<0.10$.

RESULTS AND DISCUSSION

Growth performance

The effects of BVLP on growth performance are shown in Table 4. There were no significant differences in final body weight, weight gain, feed intake, or feed conversion ratio in response to the 0.2, 0.4 and 0.8% BVLP and antibiotic treatments ($P>0.05$). In a previous study, Sarker et al. (2010a) found that the same levels of BVLP had no effect on weight gain in Ross broilers. Pollmann et al. (1980) and Kim et al. (1993) reported that microbial additives had little effect on the feed provided to finishing pigs. In general, in the animal industry, the benefits of herbs and other plant extracts can help improve the amount of feed intake, digestive enzymes, and immunity (Wenk, 2003). Tsinas et al. (1998) found that the addition of oreganum essential oil led to increased feed intake and feed conversion ratio relative to that of a control group.

Table 3. Chemical composition of experimental feed for finishing pigs (%).

Parameter	Control	Antibiotics	BVLP[1)]		
			0.2%	0.4%	0.8%
Moisture	11.36 ± 0.05	11.72 ± 0.05	11.54 ± 0.04	11.43 ± 0.07	11.60 ± 0.04
Crude protein	17.91 ± 0.33	16.15 ± 0.65	16.77 ± 0.26	17.20 ± 0.64	15.52 ± 0.14
Crude fat	5.24 ± 0.08	5.88 ± 0.16	4.65 ± 0.24	5.58 ± 0.18	5.34 ± 0.28
Crude fiber	4.37 ± 0.27	4.54 ± 0.26	4.62 ± 0.27	4.39 ± 0.03	4.83 ± 0.27
Crude Ash	5.09 ± 0.23	4.00 ± 0.13	4.88 ± 0.19	4.95 ± 0.17	4.71 ± 0.35
NFE[2)]	56.03 ± 0.43	57.70 ± 0.36	57.54 ± 0.11	56.45 ± 0.87	58.00 ± 0.05

Values are mean ± standard error and within the same row are not significantly different (P>0.05). [1)]BVLP: Bamboo vinegar liquid probiotics; [2)] NFE: nitrogen free extract

Table 4. Effects of dietary BVLP on the growth performance of finishing pigs.

Parameter	Control	Antibiotics	BVLP[1)]		
			0.2%	0.4%	0.8%
Initial weight (kg)	59.68 ± 1.16	62.02 ± 1.16	59.36 ± 1.51	58.71 ± 2.93	57.05 ± 1.60
Final weight (kg)	105.85 ± 1.39	106.36 ± 1.39	102.69 ± 1.87	104.67 ± 1.30	104.21 ± 2.33
Feed intake (kg)	146.53 ± 2.91	135.10 ± 2.91	149.00 ± 10.56	140.72 ± 3.57	133.48 ± 5.87
Weight gain (kg)	46.17 ± 2.28	44.34 ± 2.28	43.33 ± 1.10	45.95 ± 1.84	47.16 ± 1.06
FCR (Feed : Gain)[2)]	3.20 ± 0.23	3.07 ± 0.19	3.45 ± 0.33	3.08 ± 0.20	2.83 ± 0.08

Values are mean ± standard error and within the same row are not significantly different (P>0.05). [2)]FCR: Feed conversion ratio; [1)]BVLP: bamboo vinegar liquid probiotics.

Table 5. Effects of dietary BVLP on carcass grades of finishing pigs.

Parameter	Control	Antibiotics	BVLP[1)]		
			0.2%	0.4%	0.8%
Carcass weight (kg)	82.33± 1.46	79.20± 1.35	77.92± 1.61	80.10± 2.01	80.07± 1.16
Backfat thickness (mm)	23.58± 1.18	23.27± 1.08	21.00± 1.13	22.60± 0.76	21.43± 0.97
Carcass yield grade[2)]	4.42± 0.23	3.93± 0.25	3.75± 0.25	4.10± 0.35	4.21± 0.26

Values are mean ± standard error and within the same row are not significantly different (P>0.05). Carcass Yield Grade: A-5, B-4, C-3, D-2; [1)] BVLP: bamboo vinegar liquid probiotics.

Carcass grade

The effects of BVLP on the carcass grade of finishing pigs are shown in Table 5. The values of carcass weight, back fat thickness and carcass yield grade did not differ significantly, although a decreasing trend in backfat thickness was observed in 0.2% BVLP treated groups (P<0.10). There is a negative association between carcass quality grade and carcass yield grade to some extent (Moon et al., 2003). However, higher backfat thickness indicated better carcass grade according to Korean grading system (Moon et al., 2006); while for lean meat demand, lower backfat thickness is the desired criteria for the consumers based on the US grading system (Boleman et al., 1998).

Fecal microbiology

E. coli is a rod shaped, Gram-negative, facultative anaerobe, lactose-fermenting, and non–endospore-forming microorganism. *E. coli* is the most common intestinal bacterium of the Enterobacteriaceae family and its presence outside the intestine is often used as an

Table 6. Effect of dietary BVLP on fecal microbiology in finishing pigs (log_{10}cfu/g).

Microbiology	Control	Antibiotics	BVLP[1)]		
			0.2%	0.4%	0.8%
E. coli	6.67[a] ±0.36	5.18[b]±0.31	5.27[b]±0.38	5.09[b]±0.35	5.4[b]±0.25
Salmonella	7.46±0.81	6.20±1.05	6.03±0.99	5.79±1.01	6.26±0.95

[a,b]Means with different superscripts within the same row are significantly different (P<0.05). BVLP[1)]: Bamboo vinegar liquid probiotics.

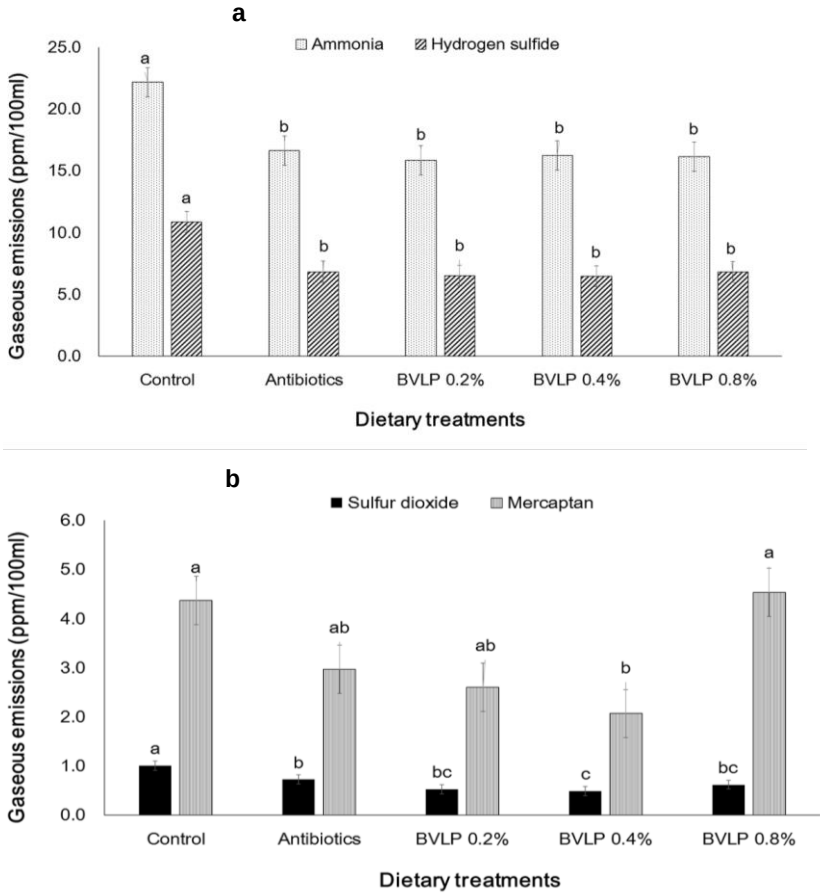

Figure 1. Effect of BVLP[1)] on fecal malodorous gas emissions (ppm/100 ml) from finishing pigs (a) ammonia and hydrogen sulfide gas; and (b) sulfur dioxide and mercaptan gas. [a,b,c]Means with different superscripts within the same bar are significantly different (P<0.05). Error bar indicated standard error. BVLP: Bamboo vinegar liquid probiotics.

indicator of fecal pollution and in surveillance programs of antimicrobial resistance (Wu et al., 2008). Screening and enumeration of antimicrobial resistant *E. coli* directly from samples is needed to identify emerging resistance and obtain quantitative data for epidemiological investigations (Aarestrup, 2004).

The effects of bamboo vinegar liquid on the fecal *E. coli* contents of finishing pigs are shown in Table 6. The *E. coli* and *Salmonella* content in feces decreased in response to all levels of BVLP and the antibiotic relative to the control. Although, the antimicrobial mechanisms are not clear, medicinal plant extracts are known to have wide antimicrobial activities. Chu et al. (2013) reported the reduction of *Salmonella* after 0.3% bamboo vinegar supplementation in fattening pigs which support our current result of all level of BVLP supplementation. Guo et al. (2004) and Lucy (2002) reported that herb polysaccharides extract decreases *E. coli* to balance the number of intestinal microbes, which is similar to the results of the present study.

Fecal malodorous gas emissions

The effects of BVLP on fecal malodorous gases emissions were measured at 21 days of the experiment are as shown Figure 1a and b. The fecal ammonia (NH_3)

and hydrogen sulfide (H_2S) were significantly lower in the antibiotic, 0.2, 0.4 and 0.8% BVLP groups than the control (Figure 1a). Where sulfur dioxide (SO_2) and mercaptan (CH_3SH) gas was significantly ($P<0.05$) lower in the 0.4% BVLP relative to control and 0.8% BVLP group and no other significant differences were observed among other groups (Figure 1b). Owing to the concern of odor from swine operations, products that potentially reduce odor emissions are being marketed. Dietary manipulation can be effective nutritional approach to reduce gaseous emissions where many factors should be considered, such as manure composition, manure pH, temperature, organic material, and antibiotics in the diets are responsible for the emissions of malodorous gas by the feces of pigs (Jongbloed et al., 1999; Sutton et al., 1999). One of the best options to reduce odor emissions are dietary manipulations that decrease nitrogen, sulfur, and phosphorus excretion is low N, Cu and Zn supplementation in the diet (Van der Peet-Schwering et al., 1999). Changing of the diet through supplementation of BVLP might be responsible for diminishing the gaseous emissions through alteration of urea concentration and pH of slurry (Van der Peet-Schwering et al., 1999). In addition, fermentable carbohydrate and crude protein content after BVLP supplementation in the diet may have played an important role in reducing the gaseous emission from the slurry in the present experiment (Le et al., 2005). Since bamboo vinegar composed of acetic acid and phenolic compounds, therefore, the higher level of BVLP might trigger to increase the volatile fatty acids rather than decrease, by altering the pH and enzymatic actions (Lin et al., 2008; Chu et al., 2013); which was opined to be the reason of difference of SO_2 and CH_3SH between higher and lower level of BVLP in the current study. Higher level of BVLP increases the chance of availability of benzoic acid, which can interact with other dietary nutrients (methionine and sulfur) and increase the SO_2 and CH_3SH in comparison with the lower level of BVLP (Eriksen et al., 2010).

Conclusion

There were no significant differences in weight gain, feed intake and feed conversion efficiency of finishing pigs observed in response to the dietary supplementation of antibiotic and BVLP. In addition to that, the carcass weight and carcass yield grade did not differ significantly among the dietary treatments. However, backfat thickness tended to be decreased in the 0.2% BVLP supplemented group in comparison with control. Furthermore, fecal microbial analysis indicated that, fecal *E. coli* content was significantly suppressed after antibiotic and 0.2, 0.4 and 0.8% BVLP supplementation relative to the control. Furthermore, among malodorous gases, ammonia and hydrogen sulfide gas was significantly down trended in the antibiotic and 0.2, 0.4 and 0.8% BVLP supplemented groups, whereas no significant differences were observed between antibiotic and BVLP supplemented groups. Significant diminution of the sulfur dioxide was found after antibiotic and 0.2, 0.4 and 0.8% BVLP supplementation as compared to the control, and the antibiotic and 0.4% BVLP group differed significantly. Moreover, the mercaptan gas was substantially decreased in the 0.4% BVLP supplemented group relative to the control and no significant differences were observed between the antibiotic and BVLP groups. In general, the current results indicated that BVLP as a dietary supplement has the potential to reduce the pathogenic fecal *E. coli* and fecal odorous gas emissions of finishing pigs without a significant negative impact on the performance. Further detailed research is required to observe the impact of BVLP on meat quality parameters.

Conflict of Interests

The authors have not declared any conflict of interests.

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