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Prevalence of extended-spectrum ß-lactamase-producing *Escherichia coli* in agricultural fields of Gorgan district using bovine manure

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ABSTRACT

The aim of this study was to assess the prevalence of ESBL-producing *Escherichia coli* in agricultural fields of Gorgan district, Golestan province. Soil samples were collected from 20 agricultural fields, half of them treated with chemical fertilizers and the rest with bovine manure. Two samples, from surface and a depth of 15cm, were taken. After enrichment and culture onto MacConkey agar, Pink colored colonies were tested for *E. coli*. The cefotaxime resistant *E. coli* were examined by phenotypic confirmatory tests. Then, antibiotic susceptibility of the confirmed ESBL-producing *E. coli* strains was determined. The results of this study showed that the prevalence of *E. coli* in the manure-amended soils were higher than the soils enriched with chemical fertilizers. Moreover, *E. coli* strains in the depth samples were more than the surface samples. About 53.1% of the strains isolated from the manure-amended soils were resistant to Cefotaxime and the 43% of them were positive for ESBL. Meanwhile, 33.3% of the strains isolated from the soils treated with chemical fertilizers were resistant to Cefotaxime in which 75% of them were ESBL producers. The highest resistance rate was observed against amoxicillin in all the ESBL-producing isolates. The results of this study showed that the use of animal manure may increases the dissemination of the antibiotic resistant bacteria to the soil. This may transfer antibiotic-resistant genes to other bacteria, causing spreading of these genes in the environment.

1. Introduction

Over the past decades, animal manure has been used to improve soil structure and fertility of agricultural fields; because of their low price and adaptability with environment. In some developing countries the animal manure, which contain animal intestinal pathogens, is applied directly into the fields without any composting process. Of course, the number existing pathogens in the animal manure depends on the source of manure, health status of animal and treatment way of manure before use (Joel V. Gagliardi, 2000).

However, pathogenic and drug-resistant bacteria such as extended-spectrum beta-lactamases (ESBLs)-producing enter to the soil and water reservoirs. Jamieson et al. studied survival and transport of enteric bacteria within and through agricultural soils (Jamieson et al., 2002). As a result, these bacteria could enter into

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other environment and food chain through different ways and threat public health.

The abundance of drug-resistant bacteria and the frequency of antibiotic resistance genes in soil and compost treated with animal manure increases. In addition, the antibiotic resistance genes can transmit between the same species or different ones through mobile genetic elements such as plasmids especially between Enterobacteriaceae.

In resent years, ESBLs-producing Enterobacteriaceae are increasing rapidly worldwide particularly ESBL-producing *E. coli* (Gao et al., 2015).The ability of ESBL-producing *E. coli* to transmit from animal farms to the surrounding environments have been shown (Laube et al., 2014; Von Salviati et al., 2015; Zhang et al., 2015). They can survive in various natural environments such as soils and water bodies (Blaak et al., 2014; Haque et al., 2014; Koczura et al., 2012). Of course, their survival in manure-amended soil depends on the manure-to-soil ratio, soil temperature, and indigenous microorganisms of the soil (Jiang et al., 2002).

To trace the spread of antibiotic resistance gene, the ESBL-producing *E. coli* is typically considered as an indicator bacterium (Gao et al., 2014). Previous studies have shown that manure application contributed to the accumulation of resistance genes in soil (Zhou et al., 2010; Heuer et al., 2011; Sengeløv et al., 2003). At present, numerous studies have focused on the occurrence and spread of ESBL genes in the environments and food-producing animals (Agerso et al., 2012; Edelstein et al., 2003).

Today, concerning about dissemination and survival of drug-resistant bacteria in the agricultural fields following extensive application of the animal manure has raised. It is therefore of importance to study on the impact of animal manure application in the agriculture fields.

Although it has received great attention in research but few studies have examined the prevalence and dissemination of drug-resistant enteric bacteria in the agricultural fields where the animal manure is applied as soil amendment. Thus the aim of this study was to evaluate the prevalence and dissemination of ESBL-producing *E. coli* in agricultural fields of Gorgan district, Golestan province.

2. Materials and Methods

2.1. Sampling

The sampling was performed in the agricultural fields of Alloukalate village, located in Gorgan district. Soil samples were collected from 20 agricultural fields that half of them had been treated with chemical fertilizer. For sampling, each of the fields was divided into four equal portions. Then from each of the portions, two samples one from the surface and the other from a depth of 15cm were taken. To remove the disturbing factor of fertilizer application timing on the study result, sampling was performed at the time of harvest.

2.2. Isolation and identification

About 0.5 g sample soil was dissolved in 5ml phosphate buffered saline. After homogenization, 1 ml of solution was mixed with 9 ml BHI broth for enrichment at 37℃ overnight. Then 0.1 ml soil enrichment was spread onto MacConkey agar plates and then incubated overnight at 37℃.

A presumptive *E. coli* colony with pink color was identified by microbiological methods and biochemical tests including Catalase and Oxidase tests, IMViC, TSI, SIM, and lysine decarboxylase, nitrate reduction, urea hydrolysis and EMB agar.

2.3. Isolation of ESBL-producing E. coli

Disk diffusion method and cefotaxime (CTX, 30 µg) was used to isolate ESBL-producing *E.coli* strains according to the Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines.

2.4. Confirmation of ESBL-producing E. coli

The screened ESBL-producing *E. coli* isolates were further confirmed by phenotypic confirmatory tests using cefotaxime (30 µg) and cefotaxime+clavulanic acid (30 µg/10 µg) according to the CLSI guidelines.

2.5. Antimicrobial susceptibility

The confirmed ESBL-producing *E. coli* isolates were subjected to antibiotics disks according to the CLSI guidelines to determine their resistance antibiotic pattern against amoxicillin (AML), Chloramphenicol (C), nalidixic acid (NA), gentamicin (GM), tetracycline (TE), imipenem (IPM). Cotrimoxazole (SXT) and ceftazidime (CE) on Mueller-Hinton agar.

3. Results

80 samples of the surface and depth of each soil samples were collected in which 49 and 12 samples were positive for *E. coli* in respect. Table 1-1 shows the absolute and relative frequency distribution of *E. coli* strains isolated from two different soils based on the enrichment with manure or fertilizer.

The number of *E. coli* strains resistant to cefotaxime in the manure-amended soils was more than the enriched one with the chemical fertilizers. Based on the results, 53.1% of the *E. coli* strains isolated from the manure-amended soils were resistant to cefotaxime meanwhile only 33.3% of the *E. coli* strains isolated from the soils treated with chemical fertilizers were resistance to cefotaxime. This difference has been statistically significant (P value <0.05) (Table 1-2).

Out of 26 cefotaxime resistant strains isolated from the manure-amended soils, 21 isolates were ESBL-producing *E. coli*. However, out of 4 cefotaxime resistant strains isolated from the soil treated with chemical fertilizer, 3 isolates were positive for ESBL.

The 21 ESBL-producing *E. coli* from the manure-amended soils showed multiple drug resistance to antibiotics tested and had above 60% resistance to AML, C, TE, NA, SXT and CE. The isolates from the soil treated with chemical fertilizer were all 100% resistant to AML and GM. They had above 60% resistance to C, SXT and CE but susceptible to IMP (Table 1-3).

Table 1. Absolute and relative frequency distribution of *E. coli* strains isolated from two different soil samples based on the enrichment with manure or fertilizer.

relative frequency	Absolute frequency	Number of samples	Sampling site	Soil samples
37.5%	15	40	Surface	Manure-amended soil
85%	34	40	Depth	
61.1%	49	80		Total number of samples
2.5%	1	40	Surface	Fertilizer-amended soil
27.5%	11	40	Depth	
15%	12	80		Total number of samples

Table 2. Absolute and relative frequency distribution of CTX resistant *E. coli* isolates

relative frequency	**Absolute frequency**	**Number of positive samples**	**Sampling site**	**Soil samples**
40%	6	15	Surface	Manure-amended soil
58.8%	20	34	Depth	
53.1%	26	49		Total number of samples
100%	1	1	Surface	Fertilizer-amended soil
27.3%	3	11	Depth	
33.3%	4	12		Total number of samples

Table 3. Frequency distribution of antibiotic resistance among ESBL-producing *E. coli* isolates

Isolates of the soil treated with chemical fertilizer			Isolates of the soil enriched with manure			Antibiotic
Susceptible No (%)	Intermediate No (%)	Resistant No (%)	Susceptible No (%)	Intermediate No (%)	Resistant No (%)	
0	0	3 (100)	3 (14.2)	1 (4.8)	17 (81)	AML
0	1 (33.3)	2 (66.7)	3 (14.2)	2 (9.5)	16 (76.3)	C
1 (33.3)	1 (33.3)	1 (33.3)	5 (23.8)	2 (9.5)	14 (66.7)	NA
0	0	3 (100)	10 (47.6)	4 (19)	7 (33.4)	GM
1 (33.3)	1 (33.3)	1 (33.3)	4 (19)	2 (9.5)	15 (71.5)	TE
1 (33.3)	0	0	19 (90.5)	1 (4.8)	1 (4.8)	IMP
1 (33.3)	0	2 (66.7)	6 (28.6)	2 (9.5)	13 (61.9)	SXT
0	1 (33.3)	2 (66.7)	5 (23.8)	1 (4.8)	15 (71.5)	CE

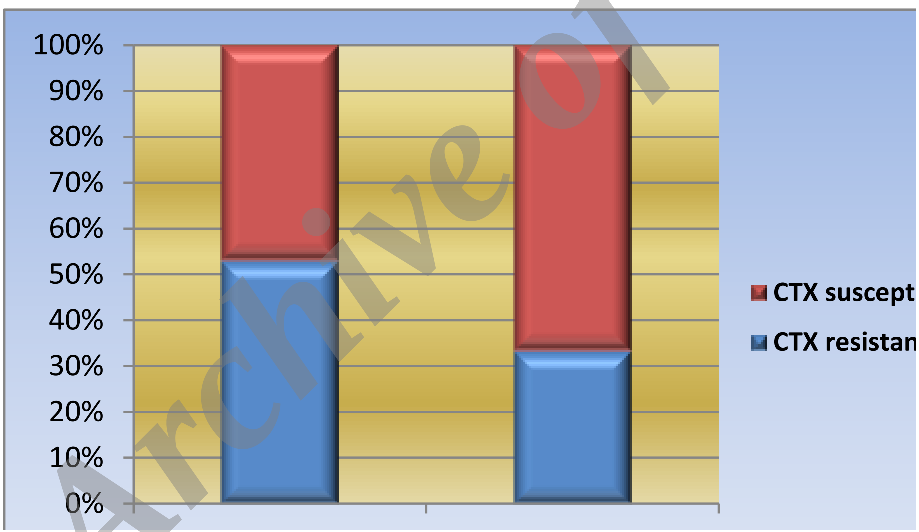

Figure 1. Absolute and relative frequency distribution of CTX resistant *E. coli* isolates

4. Discussion

Today, growing use of the animal manure and irrigation farms with sewage has increased concerns regarding pollution of the groundwater and agricultural products to a variety of pathogenic bacteria, particularly antibiotic-resistant pathogens. According to the Golestan province is one of the most important location for agriculture and animal husbandry, the aim of this research was evaluation of the antibiotic resistance pattern and the frequency of the ESBL-producing *E. coli* strains isolated from the agricultural soils around the Gorgan district.

The results of this study showed that the prevalence of isolated *E. coli* from in the soils enriched by the animal manure were more than the soils treated by chemical fertilizers that such

a result was not far from expected. In both types of the examined soils, the number of *E. coli* strains in the depth was more than the surface.
As well, we examined the resistance of isolated *E. coli* to cefotaxime. The results showed the ratio of the cefotaxime resistant strains in the manure-amended soils was much more than the soil enriched with chemical fertilizer. Based on the results, 53.1% of the *E. coli* strains isolated from the manure-amended soils showed resistance to cefotaxime, while in the case of the *E. coli* strains isolated from the soil treated with chemical fertilizers it was 33.3%.

In order to examine the ESBL production by the cefotaxime resistant isolates, confirmation test was performed. Based on the results, the prevalence of the *E. coli* strains isolated from the manure-amended soils was 43% but it was 75% for the *E. coli* strains isolated from the soil treated with chemical fertilizer.

It must be explained that sampled agricultural lands, treated with chemical fertilizer and enriched with animal manure, had a common border. Thus, the presence of ESBL strains in soil of agricultural lands treated with chemical fertilizer may be due to the water being driven to the other side.

The result of antimicrobial susceptibility showed that ESBL-producing *E. coli* isolates from the manure-amended soils were multiple drug resistance and had above 60% resistance to the most of tested antibiotics; meanwhile the isolates from the soil treated with chemical fertilizer were all 100% resistant to AML and GM and showed above 60% resistance to C, SXT and CTX but susceptible to IMP.

According to the achieved studies it is known that microbial community is affected by the type of applied amendment. Based on the report of Kirk et al. the number of cultivable bacteria in an enriched soil with organic matter and chemical fertilizer was 2 to 6 times more than the soil which had been treated only with chemical fertilizer (Kirk et al., 2004). In our study the number of *E. coli* in the manure-amended was also 6 times more than the soil treated only with chemical fertilizer. Smith and Pedrek reported the use of animal manure as a soil enrichment can lead to enteric pathogens entering water used for drinking and irrigation (Smith and Perdek, 2004). Ohtomo et al studied the survival of *E. coli* strains in soil and their results showed the survival *E. coli* strains in moist soil was more than dry ones (Ohtomo et al., 2004). Jamieson et al. studied the persistence of fecal bacteria in agricultural soils and factors affecting their survival such as moisture, temperature, pH and etc (Jamieson et al., 2002).

Gao et al performed a research about contribution of swine manure on agricultural fields to spread ESBL-producing Escherichia coli in Tai'an, China and reported that application of animal manure carrying drug-resistant bacteria on agricultural fields is a likely contributor to antibiotic resistance gene spread (Gao et al., 2015). Blaak et al in a study described the isolation of ESBL-producing *E. coli* from two poultry farms, offering a potential alternative route of transmission of ESBL-producing *E. coli* from poultry to humans by using their waste as a manure (Blaak et al., 2014). In a research, Laube et al proved transmission of ESBL/AmpC-producing *E. coli* from broiler chicken farms to surrounding areas (Laube et al., 2014). Von Salviati et al in a study reported contaminated slurry was the major emission source for ESBL/AmpC-producing *E. coli* in the pig fattening farms (Von Salviati et al., 2015).

As a result, by application of animal manure to the agricultural fields the spread of antibiotic resistant bacteria to the surrounding environment e.g. soil and water resources is possible and they may transfer antibiotic resistance genes into other bacteria such as soil bacteria; as a result these genes can be easily disseminated in the environment and threat public health.

Conclusion

In this study, we concluded the prevalence of *E. coli* strains and ESBL-producing *E. coli* in the manure-amended soils was more than in soil amended with chemical fertilizer. Moreover, the result of antimicrobial susceptibility showed that ESBL-producing *E. coli* isolates from the manure-amended soils were multiple drug resistance and had above 60% resistance to the most of tested antibiotics.

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Refereces

Blaak, H., de Kruijf, P., Hamidjaja, R.A., et al. 2014. Prevalence and characteristics of ESBL-producing E. coli In Dutch recreational waters influenced by wastewater treatment plants. Vet.Microbiol. 171:448-459.

Gao, L., Hu, J., Zhang, X., et al. 2014. Dissemination of ESBL-Producing Escherichia coli of Chicken Origin to the Nearby River Water. J.Mol.Microbiol.Biotechnol. 24:279–285.

Haque, A., Yoshizumi, A., Saga, T., et al. 2014. ESBL- producing Enterobacteriaceae in environmental water in Dhaka, Bangladesh. J.Infect.Chemother. 20:735–737.

Heuer, H., Schmitt, H., and Smalla, K. 2011a. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr.Opin.Microbiol. 14. 236–243.

Heuer, H., Solehati, Q., Zimmerling, U., et al. 2011b. Accumulation of sulfonamide resistance genes in arable soils due to repeated application of manure containing sulfadiazine. Appl.Environ. Microbiol. 77: 2527–2530.

Gagliardi, J.V., Karns, J.S., 2000. Leaching of Escherichia coli O157:H7 in Diverse Soils under Various Agricultural Management Practices. Appl.Environ. Microbiol. 4: 877–883.

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., et al. 2004. Methods of studying soil microbial diversity. J Microbiol Meth. 59:169-188.

Koczura, R., Mokracka, J., Jablonska, L., Gozdecka, E., et al. 2012. Antimicrobial resistance of integron-harboring Escherichia coli isolates from clinical samples, wastewater treatment plant and river water. Sci. Total Environ. 414: 680–685.

Laube, H., Friese, A., von Salviati, C., et al. 2014. Transmission of ESBL/AmpC-producing Escherichia coli from broiler chicken farms to surrounding areas. Vet. Microbiol. 172: 519–527.

Gao, L., Hu, J., Zhang, X., Wei, L., Song, Li., et al. 2015. Application of swine manure on agricultural fields contributes to extended-spectrum b-lactamase-producing Escherichia coli spread in Tai’an, China". Front. Microbiol. 6: Article 313.

Jamieson, R.C., Gordon, R.J., Sharples, K.E., et al. 2002. Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. Can. Biosyst. Eng., 44: 1-8.

Sengeløv, G., Agersø, Y., Halling Sørensen, B., Baloda, S.B., et al. 2003. Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. Environ.Int. 28:587–595.

Ohtomo, R., Minato, K., Saito, M. 2004. Survival of Escherichia coli in a field amended with cow feces slurry. Soil Science and Plant Nutrition. 50(4):575-81.

Smith, J., Perdek, J. 2004. Assessment and management of watershed microbial contaminants. Crit. Rev. Environ. Sci.Technol. 34:109-139.

Von Salviati, C., Laube, H., Guerra, B., et al. 2015. Emission of ESBL/AmpC producing Escherichia coli from pig fattening farms to surrounding areas. Vet.Microbiol. 30: 77–84.

Zhang, H., Zhou, Y., Guo, S., and Chang, W., 2015. Multidrug resistance Found in extended-spectrum beta-lactamase-producing Enterobacteriaceae From rural water reservoirs in Guantao, China. Front.Microbiol. 6:267

Zhou, Z., Raskin, L., and Zilles, J.L. 2010. Effects of Swine manure on macrolide,lincosamide,and streptogramin B antimicrobial resistance in soils. Appl.Environ.Microbiol. 76: 2218–2224.

Jiang, X., Morgan, J. and Doyle, M.P. 2002. Fate of Escherichia coli O157:H7 in Manure-Amended Soil. Appl.Environ. Microbiol. 5: 2605–2609.