IJMPR 2020, 4(5), 99-110

International Journal of Modern Pharmaceutical Research
www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article
SJIF Impact Factor: 5.273

ANTIBIOTICS RESISTANCE PROFILE OF BACTERIAL ISOLATES FROM DAIRY FARMS MANURE IN BAHRI LOCALITY, SUDAN

Sarah Azhary Mohamed[1], Yassir A. Almofti*[2], Elniema A. Mustafa[3] and Sheimaa S. A. O. Fagirii[4]

[1,3]Department of Food Safety and Veterinary Public Health, College of Veterinary Medicine, University of Bahri, Khartoum- Sudan.
[2]Department of Molecular Biology and Bioinformatics, College of Veterinary Medicine, University of Bahri, Khartoum- Sudan.
[4]Department of Microbiology, College of Veterinary Medicine, University of Bahri, Khartoum- Sudan.

Received on: 29/08/2020
Revised on: 09/09/2020
Accepted on: 19/09/2020

***Corresponding Author**
Almofti Y. A.
Department of Molecular Biology and Bioinformatics, College of Veterinary Medicine, University of Bahri, Khartoum- Sudan.
yamofti99@gmail.com

ABSTRACT

This study was conducted between November 2016 and February 2018 and aimed to isolate some bacteria, mainly *E. coli, Staphylococci* and *Salmonella* from dairy farms manure and to assess their antibiotics resistance profile to different antimicrobial agents. This study included 19 dairy farms distributed in Bahri locality, Khartoum, Sudan. A total of 95 manure samples were collected from these farms and a questionnaire was introduced to each dairy farm owner before samples collection. The questionnaire showed that the density of the cows within the farms was appropriate. Mastitis was common in visited farms and the control of mastitis was made by the owners. The majority of the farms had no hoof care and the incidence of lameness was frequent. Also multiple diseases such as abortion, metritis, retention of placenta, pneumonia, eye infection and calf diarrhea that requires antibiotic treatment were prevalent in the visited farms. The majority of the cows received at least two courses of antibiotics treatment per year. In addition to that the most commonly used antibiotics were tetracycline and tylosin, penicillin and the least used antibiotic was ciprofloxacin. These antibiotics were mostly administered by the owners rather than veterinarians. A total of 68 bacterial isolates were identified using the conventional bacteriological isolation method. These include 28 isolates as *E. coli* (41.18%), 26 isolates as *Staphylococci* (38.23%) and 14 isolates as *Salmonella* (20.58%). The isolates were subjected to antimicrobial susceptibility test using broth microdilution method. The three isolates showed resistance to Erythromycin, Azithromycin and Tetracycline but demonstrated susceptibility to Ciprofloxacin. Therefore, it could be concluded that untreated manure could impose a great risk if it is used as a fertilizer in vegetables farms as the resistant bacteria in manure may transferred to humans through contaminated vegetables.

KEYWORDS: Dairy cows, manure, bacterial isolates, questionnaire, antibiotic resistance.

1. INTRODUCTION

Livestock and feedlots produced large amounts of animal manure each year from chickens and cattle farms.[1] Worldwide, studies demonstrated the total amount of animal manure reported to be 3.19 billion tons in 2003 in China.[1,2] The majority of the manure produced was utilized in agriculture.[1] Manure provides different biological and physicochemical environment to microorganisms.[3] Specifically, Pachepsky et al. (2006).[4] noted that many manure-based pathogens exist, but the major manure based zoonotic bacteria, including Salmonella spp., Campylobacter spp., Listeria monocytogenes, Yersinia enterocolitica, Escherichia coli and protozoa viz. Cryptosporidium parvum and Giardia lamblia, are present; however others are less common. Viruses represent another group of pathogens that exist in cattle manure. Originally, these pathogens inhabit the intestinal tracts of animals and are typically shed in this habitat asymptomatically.[5] Seemingly, both animals and humans on and off farms are exposed to the potential health risks allied to inadequate management of manure. Consequently, the fate of these pathogens in manure is to pollute, contaminate and infect the environment and humans, based on the pathogen's ability to survive in manure following excretion.[3] Nevertheless, the factors affecting the survival rates of these well-documented pathogens excreted in cattle manure have been enumerated and deliberated in many reports.[3-5]

Manure from dairy cows, which commonly used as a farm soil fertilizer, contains a surprising number of

newly identified antibiotic resistance genes from the cows' gut bacteria.[6] Cow manure is a potential source of new types of antibiotic resistance genes that transfer to bacteria in the soils where food is grown.[7] The movement of antibiotic resistant genes to agricultural soils may be enhanced by various management practices, for instance, the application of animal manure, waste-water, or waste treatment residues that contain antibiotic resistant genes on mobile elements and antibiotic residues.[8-11]

Misuse and overuse of antibiotics in food-animal production lead to transfer of antibiotic residues, resistant bacteria and resistant genes to terrestrial and aquatic environment through farm-generated waste. Untreated manure also poses greater risk of spreading antimicrobial resistance (AMR) into the environment. There is, therefore, a need for greater focus and global guidance on addressing AMR in the environment through research-backed evidence, proper waste management, effluent treatment, biosecurity measures at all settings and appropriate regulations and policies. The aim of the present study was to isolate common bacterial species from dairy farm untreated manure mainly E. coli, Staphylococci and Salmonella and identify the antibiotic resistant profile of these bacterial isolated.

2. MATERIAL AND METHODS

2.1 Study area

This study was conducted between November 2016 and February 2018 in Bahri locality in Khartoum State. It included 19 dairy farms in Alhaj Yousif, Alkadaro, Shambat and Alhalfaya regions. The cows in these farms were raised in zero grazing system.

2.2 Questionnaire

Before samples collection a questionnaire was introduced to the dairy farms owners. The questionnaire mainly focused on the barns manure cleanness, the common diseases and antibiotic usage in the farms.

2.3 Samples collection

Fecal samples were collected from cow's fresh manure from different parts of each farm. About 25 grams of manure was collected in sterile plastic bag. Immediately after collection samples were transported to the laboratory of the University of Bahri, College of Veterinary Medicine at Alkadaro for bacteriological investigation.

2.4 Bacteriological investigation

Manure from the dairy farms contains multiple bacterial species. This study aimed to isolate the common bacterial species in cow manure, mainly, *Escherichia coli*, *Staphylococci* and *Salmonella spp*. For identification of the isolates the conventional method was used.

2.4.1 Biochemical identification of the bacterial isolates

For isolation and identification of *Escherichia coli*, *Salmonella* and *Staphylococcus aureus* from the collected samples, the samples were first enriched by incubation for 12 hours at 37°C, sub-culturing on nutrient agar plates to purify colonies followed by incubation at 37°C for another 24 hours. The colonial characteristics on selective media; Eosine Methylene Blue media Agar, Xylose-lysine Deoxycholate Agar (XLD) and Baird parker agar base, respectively was used to identify the isolates. The purified isolates were further identified according to the reaction of Gram's stain, shape of the bacterial colonies, as outlined by Barrow and Feltham, (1993).[12] Tests performed included: catalase test, coagulase test, oxidase test, indole test, citrate utilization, methyl red, motility test and kligler test.

2.5 Antibiotic sensitivity test

Minimum inhibitory concentration (MIC) of the isolated strains was determined using broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI).[13] The antimicrobials tested (Sigma–Aldrich, St Louis, MO) consisted of the following: Erythromycin (ERY), Azithromycin (AZM) in addition to Ciprofloxacin (CIP) and Tetracycline (TET). The resistant breakpoints for each antibiotic were according to the CLSI (2016). Resistant breakpoints were as following: for E. coli and Salmonella: Azithromycin ≥32 µg/ml (the resistant breakpoint of Azithromycin was used for Erythromycin ≥32µg/ml), Tetracycline ≥16µg/ml and Ciprofloxacin ≥1µg/ml. For Staphylococci the resistant breakpoints: Azithromycin ≥8µg/ml, Erythromycin ≥8µg/ml, Tetracycline ≥16µg/ml and Ciprofloxacin ≥4µg/ml.[13]

3. RESULTS

3.1 Questionnaire

The questionnaire was introduced to each farm owner and the result was shown in table (1)

Table 1: Provided the questions and the answers of the questionnaire conducted with the visited farms owners.

Feature	Result
Appropriate density of livestock	
Appropriate	(17farms) 89.5%
Crowded	(2 farms) 10.5%
Barns cleanliness (manure collection and management)	
Daily	(1 farms) 5%
3 to 4 days	(9 farms) 47.4%
Weekly	(9 farms) 47.4%
Incidence of mastitis	
Few	(3 farms) 15.8%
Many	(16 farms) 84.2%
Presence of mastitis prevention control	
By owner	(12 farms) 63.2%
By a veterinarian	(7 farms) 36.5%
Presence of hoof care	
Found	(13 farms) 68.4%
Not found	(6 farms) 36.8%
Incidence of lameness	
Found	(2 farms) 10.5%
Not found	(17farms) 89.5%
Common diseases in the farms require antibiotics treatment	
Abortion	21%
Metritis	42.1%
Retention of placenta	36.8%
Pneumonia	63.2%
Eye infection	73.7%
Calf diarrhea	94.7%
Commonly used antibiotics in the farms:	
Tetracycline	36.8%
Tylosine	31.6%
Penicillin	21%
Ciprofloxacin	10.5%
Who administered these medications?	
Owner	73.7%
Veterinary doctors	26.3%

3.2 Bacteriological identification

The bacteriological identification of the bacteria isolated from manure was shown in figures (1, 2, 3, 4 and 5).

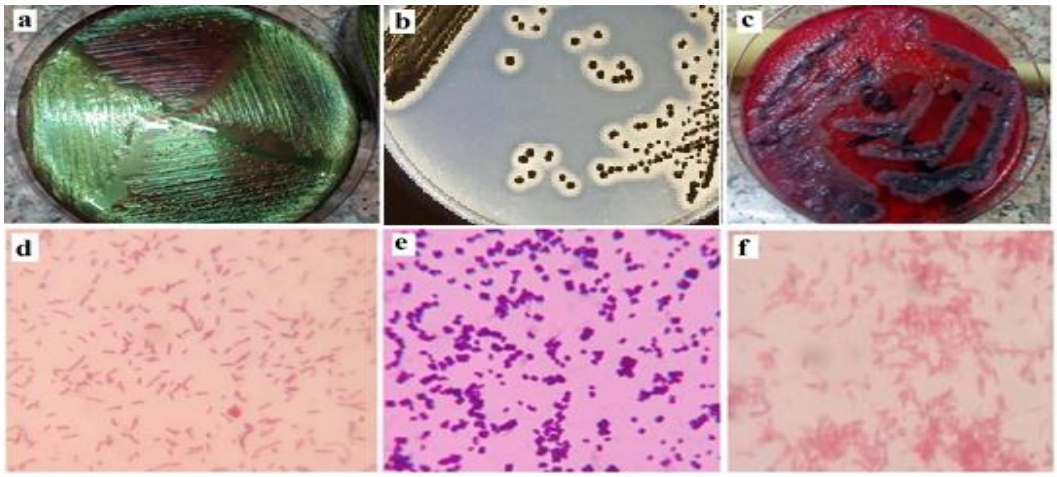

Figure (1): The growth of the bacterial isolates in selective media and gram staining: (a) EMB, selective media: the metallic shining growth on EMB media indicated the colonies as E. coli. (b) Baired parker, selective media: the black colonies and digestion of protein egg with clear zone around the colonies indicated isolates as

Staphylococci. (c) XLD selective media: raised, circular smooth, glistening, opaque, red colonies with black center indicated the colonies as Salmonella. One colony from each selective media was prepared for gram staining. (d): showed gram negative rods, (e): showed gram positive clustered coccids and (f): showed gram negative rods. Thus (d, e and f) indicating isolates as E. coli, Staphylococci and Salmonella, respectively.

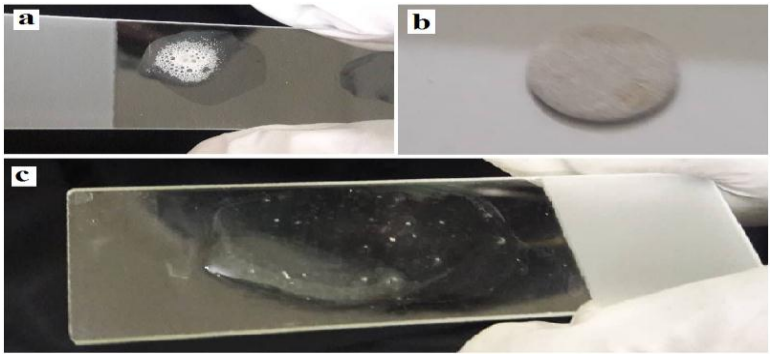

Figure (2): Catalase, oxidase and coagulase tests: (a) both E. coli and Staphylococci demonstrated gas bubbles with catalase (positive for catalase), while Salmonella showed no gas bubbles (negative for catalase). (b): oxidase test: the three isolates demonstrated no violet color with oxidase which indicated the isolates were negative for oxidase. (c): Coagulase test: this test was performed only for Staphylococci and a milky suspension was made indicating positive for Staphylococci.

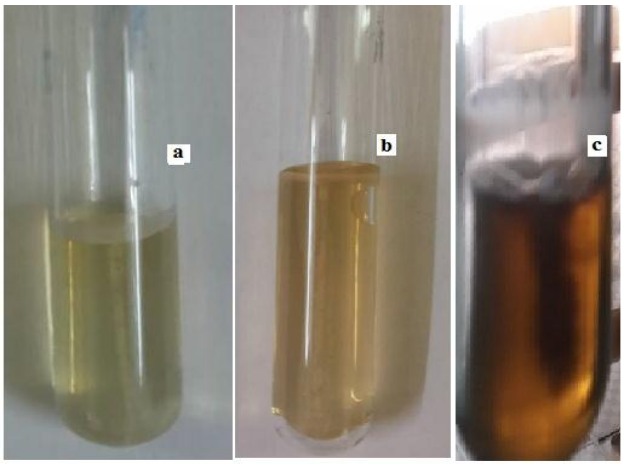

Figure (3): The motility test of the three isolates. (a): the motility of the E coli was shown to be motile. (b): Staphylococci showed no turbidity in the media thus it was non motile. (c): Salmonella showed obvious turbidity thus it was motile.

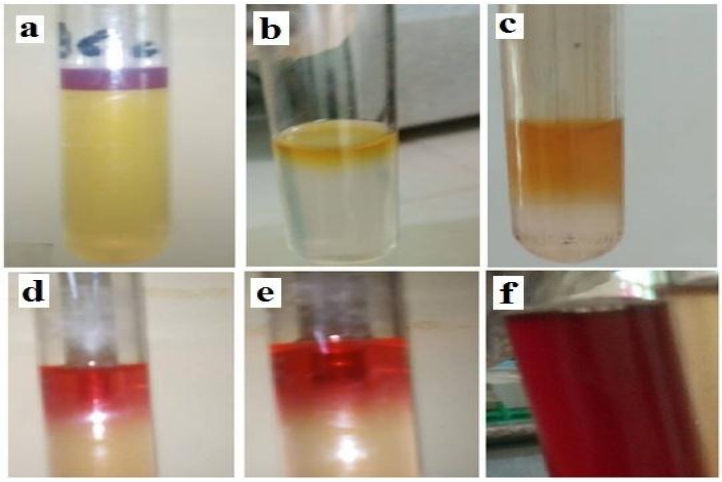

Figure (4): Indole and methyl red tests: (a):only E. coli was positive for the indole test since pink color appeared on the top of the tube, while Staphylococci (b) and Salmonella (c) were negative for indole. However, the three isolates (d, e, f) all were positive for VP test since the presence of red colors were observed in all tubes.

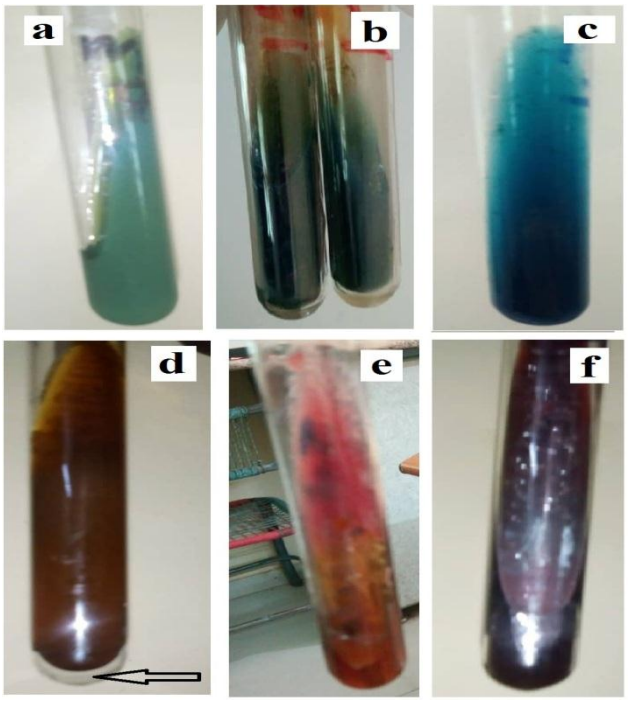

Figure (5): citrate utilization and Kligler tests: (a) E coli and (b) Staphylococci showed negative results since light green color was made. However, Salmonella (c) was shown to be positive for citrate utilization as blue color was obtained. In Kligler test E coli (d) showed brown color with gas production (indicated by an arrow), Staphylococci (e) showed light brown color and Salmonella (f) provided black color due to the presence of H_2S. Colors obtained by each bacterium were indicative for each isolate.

3.3 Bacterial isolates

The number of the visited farms, samples collected from each farm and the numbers of the bacterial isolates were demonstrated in table (2). The table shows that a total of 19 dairy farms were visited and 95 manure samples were collected. From these samples 68 bacterial isolates were identified.

Table 2: The number of the visited farms, samples collected and identified isolates.

Region	Number of the visited farms	Samples collected from each farm	Total samples collected	Isolated Bacteria			Total of the isolates
				E. coli	*Staphylococci*	*Salmonella*	
Alhaj Yousif	4	5	20	5	4	2	11
Shambat	4	5	20	5	5	3	13
Alhalfaya	5	5	25	10	5	3	18
Alkadaro	6	5	30	8	12	6	26
TOTAL	**19**	**20**	**95**	**28**	**26**	**14**	**68**

As shown in table (2) out of 95 fecal samples collected from different regions in Bahri locality (Alhaj Yousif, Shambat, Alhalfaya and Alkadaro) 68 bacterial isolates were identified using the conventional isolation method and the result was provided in table (3). The identified isolates were 28 isolates (41.18%) as E coli, 26 isolates (38.23%) as Staphylococci and 14 isolates (20.58%) as Salmonella.

Table (3): Conventional methods used for identification of bacterial isolates.

Characteristics/organism*	E. coli	Staphylococci	Salmonella
Colonial characteristics	2-3mm flat, metallic shining in Eosin Methylene Blue	Round 1-2 mm diameter, black in Baired parker agar media	raised, opaque red colonies with black center in XLD
	Lactose fermenter in McConkey		
Gram's stain	-ve bacilli	+ve cocci/ cluster	-ve bacilli
Colony shape	Flat 2-3 mm	Round smooth	Smooth mucoid
Methyl red	+ve	+ve	+ve
Motility	Majority Motile	Non Motile	Motile
Catalase	+ve	+ve	-ve
Oxidase	-ve	-ve	-ve
Coagulase	-	+ve only in pathogenic species	-
Indole test	+ve	-ve	-ve
Citrate utilization	-ve	-ve	+ve
Kligler	Black color with gas production	Red or purple color	Black color H2S production

*The biochemical characteristics of each bacterial isolates in this study (+ve: positive; -ve: negative; (-) not assessed.

3.4 Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed using broth microdilution method recommended by.[13]

3.4.1 Antibiotics susceptibility test for Alhaj Yousif region isolates

Table (4) provided five isolates as E. coli, two isolate as Salmonella and four isolates as Staphylococci. The five isolates of E. coli demonstrated 100% resistance to Erythromycin and Azithromycin (the breakpoint was ≥32µg/ml). However, three isolates showed resistance to Tetracycline (≥16µg/ml) with resistance profile (R) 60%. In case of Ciprofloxacin, four isolates of E. coli demonstrated susceptibility to the drug and only one isolate demonstrated resistance to Ciprofloxacin with resistance profile (R) 20%. For Salmonella, the two isolates demonstrated 100% resistance to Erythromycin and Azithromycin (the breakpoint was ≥32µg/ml). However, one isolate showed resistance to Tetracycline (≥16µg/ml) with resistance profile (R) 50%. In case of Ciprofloxacin, one isolate demonstrated susceptibility to the drug and only one isolate demonstrated resistance with resistance profile (R) 50%.

Table (4): Antimicrobial susceptibility test for the bacterial isolates of Alhaj Yousif region.

Region	Bacterial isolate	Antimicrobial susceptibility µg/ml			
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
Alhaj yousif	E. coli 12	128	128	16	1
	E. coli 13	128	128	64	0.5
	E. coli 16	128	128	8	0.5
	E. coli 18	32	32	8	0.125
	E. coli 20	128	64	64	0.125
	Resistance profile	**R 100%**	**R 100%**	**R 60%**	**R 20%**
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
	Salmonella 6	128	128	8	0.125
	Salmonella13	64	32	32	1
	Resistance profile	**R 100%**	**R 100%**	**R 50%**	**R 50%**
		ERY≥8µg/ml	**AZM≥8µg/ml**	**TET≥16µg/ml**	**CIP≥4µg/ml**
	Staphylococci9	64	32	32	2
	Staphylococci15	32	16	32	2
	Staphylococci19	64	16	8	8
	Staphylococci16	8	4	8	2
	Resistance profile	**R 100%**	**R 75%**	**R 50%**	**R 25%**

For Staphylococci, the four isolates showed 100% and 75% resistance profile to Erythromycin and Azithromycin (≥8µg/ml), respectively. Two isolates showed susceptibility to Tetracycline (≥16µg/ml) with resistance profile of 50%. For Ciprofloxacin, three isolates were found susceptible (≥4µg/ml) and one isolate was resistance (R 25%).

3.4.2 Antibiotics susceptibility test for Alkadaro region isolates

As shown in table (5) multiple bacterial isolates for Alkadaro region were obtained. Eight isolates were identified as E. coli, six isolates as Salmonella and twelve isolates as Staphylococci. Among the eight isolates of the E. coli, one isolate showed susceptibility to Erythromycin and Azithromycin, while the other seven isolates demonstrated resistance (≥32µg/ml) to Erythromycin and Azithromycin with resistance profile of 87.5% for each. Also, one isolate showed susceptibility to Tetracycline, while the other seven isolates demonstrated resistance (≥16µg/ml) to Tetracycline with resistance profile of 87.5%. In case of Ciprofloxacin, all the eight isolates showed susceptibility to Ciprofloxacin (≥1µg/ml).

Table (5): Antimicrobial susceptibility test for the bacterial isolates of Alkadaro region.

Region	Bacterial isolate	Antimicrobial susceptibility µg/ml			
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
Alkadaro	E. coli28	32	32	32	0.125
	E. coli30	128	128	32	0.125
	E. coli10	128	128	8	0.125
	E. coli25	32	32	32	0.125
	E. coli15	16	16	16	0.125
	E. coli20	64	128	64	0.125
	E. coli4	64	32	32	0.125
	E. coli27	128	128	32	0.125
	Resistance profile	**R 87.5%**	**R 87.5%**	**R 87.5%**	**R 0%**
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
	Salmonella 10	16	128	64	0.125
	Salmonella 25	128	128	8	0.125
	Salmonella 26	128	128	32	0.125
	Salmonella 27	64	128	16	0.125
	Salmonella 9	128	128	4	0.125
	Salmonella 5	32	128	32	0.125`
	Resistance profile	**R 83.5 %**	**R 100%**	**R 66.6%**	**R 0 %**
		ERY≥8µg/ml	**AZM≥8µg/ml**	**TET≥16µg/ml**	**CIP≥4µg/ml**
	Staphylococci 12	64	128	64	4
	Staphylococci 30	8	4	16	0.125
	Staphylococci 5	8	8	8	0.125
	Staphylococci 28	8	2	64	2
	Staphylococci 13	64	64	32	0.125
	Staphylococci 27	128	128	128	0.125
	Staphylococci 11	128	128	16	2
	Staphylococci 14	128	128	128	0.125
	Staphylococci 15	64	128	16	0.125
	Staphylococci 18	8	8	16	1
	Staphylococci 17	16	8	32	0.125
	Staphylococci 8	128	128	8	0.125
	Resistance profile	**R 100%**	**R 83.3 %**	**R 83.3 %**	**R 8.3 %**

Salmonella (six isolates) five isolates showed resistance to Erythromycin with resistance profile of 83.5% and only one isolate was found susceptible. However, they all demonstrated high resistance to Azithromycin (128µg/ml) (R 100%). For Tetracycline, four isolates were found resistance (R 66.6%) and two isolates were found susceptible. The six isolates showed absolute susceptibility to Ciprofloxacin.

For Staphylococci (12 isolates), all isolates showed resistance (≥8µg/ml) to Erythromycin (R100%) and ten isolates showed resistance to Azithromycin (83.3%). For Tetracycline, also ten isolates showed resistance (R 83.3%). While eleven isolates showed susceptibility to Ciprofloxacin (≥4µg/ml) and only one was found to be resistant (R 8.3%).

3.4.3 Antibiotics susceptibility test for Shambat region isolates

As shown in table (6) the isolates from Shambat region were five isolates as E. coli, three isolates as Salmonella and five isolates as Staphylococci. All the five isolates of the E. coli showed 100% resistance to Erythromycin, Azithromycin (≥32µg/ml), Tetracycline (≥16µg/ml) and Ciprofloxacin (CIP≥1µg/ml). Also all the isolates of Salmonella showed 100% resistance to Erythromycin, Azithromycin (≥32µg/ml) and Tetracycline (≥16µg/ml).

Table (6): Antimicrobial susceptibility test for the bacterial isolates of Shambat region.

Region	Bacterial isolate	Antimicrobial susceptibility µg/ml			
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
Shambat	E. coli17	64	64	64	1
	E. coli19	128	64	16	2
	E. coli1	32	32	128	1
	E. coli 2	128	64	64	8
	E. coli20	32	32	64	8
	Resistance profile	**R 100%**	**R 100 %**	**R 100%**	**R 100%**
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
	Salmonella 2	64	128	32	16
	Salmonella 1	128	64	32	0.125
	Salmonella 7	32	32	32	8
	Resistance profile	**R 100%**	**R 100%**	**R 100%**	**R 66.6 %**
		ERY≥8µg/ml	**AZM≥8µg/ml**	**TET≥16µg/ml**	**CIP≥4µg/ml**
	Staphylococci 12	128	128	64	32
	Staphylococci 17	128	128	8	0.125
	Staphylococci 23	64	128	2	0.125
	Staphylococci 14	8	8	32	0.125
	Staphylococci 15	64	128	16	0.125
	Resistance profile	**R 100%**	**R 100 %**	**R 60%**	**R 20%**

For Ciprofloxacin one isolate showed susceptibility while two isolates were resistant (66.6%). For Staphylococci (five isolates), showed 100% resistance to Erythromycin and Azithromycin (≥8µg/ml). Two isolates showed susceptibility to Tetracycline (≥16µg/ml) and three isolates showed resistance (60%). For Ciprofloxacin, four isolates were susceptible and one was found resistance (4µg/ml) (R 20%).

3.4.4 Antibiotics susceptibility test for Alhalfaya region isolates:

Isolates from Alhalfaya region were ten isolates as E. coli, three isolates as Salmonella and five isolates as Staphylococci. As shown in table (7) the ten isolates of the E. coli showed 100% resistance to Erythromycin, Azithromycin (≥32µg/ml) and Tetracycline (≥16µg/ml).

The resistant breakpoints for each antibiotic were according to the CLSI (2016).

Table (7): Antimicrobial susceptibility test for the bacterial isolates of Alhalfaya region.

Region	Bacterial isolate	Antimicrobial susceptibility µg/ml			
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
Alhalfaya	E. coli 10	128	64	16	0.125
	E. coli 12	128	128	16	32
	E. coli 15	32	32	32	32
	E. coli 24	64	64	64	8
	E. coli 19	64	64	64	16
	E. coli 21	128	128	64	2
	E. coli 22	64	64	64	4
	E. coli 14	128	128	16	1
	E. coli 7	32	32	32	4
	E. coli 8	128	128	16	0.125
	Resistance profile	**R 100%**	**R 100%**	**R 100%**	**R 80%**
		ERY≥32µg/ml	**AZM≥32µg/ml**	**TET≥16µg/ml**	**CIP≥1µg/ml**
	Salmonella 34	32	32	32	16
	Salmonella 55	32	32	32	8
	Salmonella 77	64	64	32	8
	Resistance profile	**R 100%**	**R 100%**	**100%**	**100%**
		ERY≥8µg/ml	**AZM≥8µg/ml**	**TET≥16µg/ml**	**CIP≥4µg/ml**
	Staphylococci 24	128	128	32	8
	Staphylococci 8	32	128	64	1
	Staphylococci 21	128	128	64	16
	Staphylococci 22	8	8	32	8
	Staphylococci 25	64	64	32	8
	Resistance profile	**R 100%**	**R 100%**	**100%**	**R 80%**

However, eight isolates showed resistance to Ciprofloxacin (≥1µg/ml) (R 80%). For Salmonella, the three isolates showed 100% resistance to the all tested antibiotics (R 100% for each isolate.

For Staphylococci, the five isolates showed 100% resistance to Erythromycin, Azithromycin (≥8µg/ml) and Tetracycline. The five isolates showed resistance to Tetracycline (≥16µg/ml). Four isolates showed resistance (R 80%) to Ciprofloxacin (≥4µg/ml).

Figure (6) showed the number of the all susceptible isolates for each bacterial isolates compared to the number of the all resistance isolates in this study. As shown in the figure the grey areas indicated susceptibility while the white areas indicated resistance of the isolates.

isolates	ERY≥32µg/ml	AZM≥32µg/ml	TET≥16µg/ml	CIP≥1µg/ml
E. coli 12	128	128	16	1
E. coli 13	128	128	64	0.5
E. coli 16	128	128	8	0.5
E. coli 18	32	32	8	0.125
E. coli 20	128	64	64	0.125
E. coli28	32	16	32	0.125
E. coli30	128	128	32	0.125
E. coli10	128	128	8	0.125
E. coli25	32	32	32	0.125
E. coli15	16	32	16	0.125
E. coli20	64	128	64	0.125
E. coli4	64	32	32	0.125
E. coli27	128	128	32	0.125
E. coli17	64	64	64	1
E. coli19	128	64	16	2
E. coli1	32	32	128	1
E. coli 2	128	64	64	8
E. coli20	32	32	64	8
E. coli 10	128	64	16	0.125
E. coli 12	128	128	16	32
E. coli 15	64	32	32	32
E. coli 24	32	64	64	8
E. coli 19	64	64	64	16
E. coli 21	128	128	64	2
E. coli 22	64	64	64	4
E. coli 14	128	128	16	1
E. coli 7	32	32	32	4
E. coli 8	128	128	16	0.125

isolates	ERY≥32µg/ml	AZM≥32µg/ml	TET≥16µg/ml	CIP≥1µg/ml
Salmonella 6	128	128	8	0.125
Salmonella13	32	32	32	2
Salmonella 10	16	128	64	0.125
Salmonella 25	128	128	8	0.125
Salmonella 26	128	128	32	0.125
Salmonella 27	64	128	16	0.125
Salmonella 9	128	128	4	0.125
Salmonella 5	32	128	32	0.125
Salmonella	64	128	32	16
Salmonella 1	128	64	32	0.125
Salmonella 7	64	32	32	8
Salmonella	32	32	32	16
Salmonella	32	32	32	8
Salmonella	64	64	32	8

isolates	ERY≥8µg/ml	AZM≥8µg/ml	TET≥16µg/ml	CIP≥4µg/ml
Staphylococci9	64	32	32	2
Staphylococci15	32	16	32	2
Staphylococci19	64	16	8	8
Staphylococci16	8	4	8	2
Staphylococci 12	64	128	64	4
Staphylococci 30	8	4	16	0.125
Staphylococci 5	8	8	8	0.125
Staphylococci 28	8	2	64	2
Staphylococci 13	64	64	32	0.125
Staphylococci 27	128	128	128	0.125
Staphylococci 11	128	128	16	2
Staphylococci 14	128	128	128	0.125
Staphylococci 15	64	128	16	0.125
Staphylococci 18	8	8	16	1
Staphylococci 17	16	8	32	0.125
Staphylococci 8	128	128	8	0.125
Staphylococci 12	128	128	64	32
Staphylococci 17	128	128	8	0.125
Staphylococci 23	64	128	2	0.125
Staphylococci 14	8	8	32	0.125
Staphylococci 15	64	128	16	0.125
Staphylococci 24	128	128	32	8
Staphylococci 8	32	128	64	1
Staphylococci 21	128	128	64	16
Staphylococci 22	8	8	32	8
Staphylococci 25	64	64	32	8

Figure (6): The number of susceptible isolates compared to the number of the resistant isolates.

In addition, figure (7) showed that among all E coli isolates (28 isolates) one isolate was susceptible to Erythromycin with resistance profile R 96.43%. In addition, one isolate was susceptible to Azithromycin with resistance profile R 96.43% as well. For Tetracycline, the resistance profile was R 89.28% since only three isolates were shown to be susceptible. For Ciprofloxacin, 50% of the isolates were susceptible and thus the resistance profile was R 50%. In addition to that Salmonella isolates (14 isolates) from the four regions showed resistance to Erythromycin with resistance profile of R 93%, Azithromycin with resistance profile of R 100%, and Tetracycline with resistance profile of R 78%. However, these isolates demonstrated susceptibility to Ciprofloxacin (57%) with resistance profile of R 43%. For Staphylococci isolates (26 isolates), the resistance profile of Erythromycin, Azithromycin, Tetracycline and Ciprofloxacin was R 100 %, R 88 %, R 77% and R 27%, respectively.

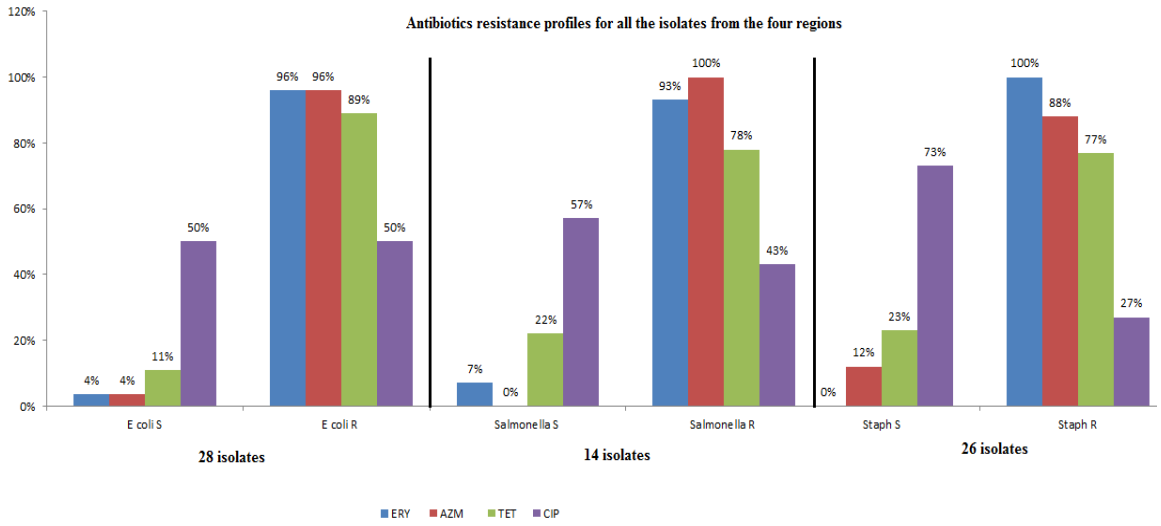

Figure (7): Antimicrobial resistant profile for all the isolates of the four regions. The (%) of the resistant profile was calculated by dividing the number of the resistant isolates to the total number of the bacterial isolates. ERY: Erythromycin; AZM: Azithromycin; TET: Tetracycline; CIP: Ciprofloxacin; S: susceptible; R: resistant.

4. DISCUSSION

Manure from dairy cows farms, which are commonly used as a farm soil fertilizer, contain a surprising number of bacteria and other organisms from the cows' gut. Moreover, this manure is a potential source of antibiotic resistance bacteria in the soils where food is grown.[14] Some manure bacteria might be pathogenic to humans, so if they acquire antibiotic resistance, they could pose a health problem. Alternatively, benign bacteria in manure might transfer resistance genes to pathogens at any point along the path-in manure, soil, food, or humans. This study investigated some bacteria present in cow manure from dairy farms in Bahri locality including E. coli, Salmonella and Staphylococci and assessing their antimicrobial resistance profile.

In this study, a survey questionnaire was conducted and it reflected the real animal welfare situation in the dairy farms concerning the cow's density within the farms, cleanness of the barns from manure, the diseases that required antibiotics treatment and the ways of antibiotic usage and administrations. The increase of cow's density within the farms could result in mass production of cow manure. In this study, the results obtained by the questionnaire concerning the amount of the manure were observed greater in the crowded farms and in those farms with long period of the barns being not cleaned. The accumulation of manure considered as suitable environment for bacterial growth.[1,2,15] In addition to that, this study clearly showed that Mastitis is the most common disease of dairy cows and the most common reason that cows are treated with antibiotics.[16] Therefore, we anticipated that mastitis contributed in the contamination of manure in farm with antibiotic resistant bacteria. In addition to mastitis, other diseases that required antibiotics treatment were recognized in the visited farms. All these diseases could contribute in one way or another in the existence of the susceptible and antibiotics resistant bacteria in manure, especially if the fluids, remnants and/ or debris from these diseases reached manure.

One important observation from the questionnaire was that farmers misused antibiotics in animal dairy farms due to their ignorance of the importance of optimal use of antibiotics, the potential health hazards and the economical waste associated with antibiotic misuse. This observation was coincided with the previously published work of imprudent usage of antibiotics in dairy farms in Khartoum state.[17,18]

To elucidate the antibiotics resistant genes or resistance profile of manure in the dairy farms, 95 samples of manure from the visited farms were collected to isolate bacteria from manure and study their antimicrobial resistance. It is quite unrealistic to enumerate all the microbial pathogens present in cattle manure because of the huge numbers of these pathogens in the cow gut. This may be linked to the limitations in some of the available methods for identification of these pathogens. Furthermore, some pathogens require time-intensive tests and enrichment steps during analysis and detection, thereby making their quantification complex.[19] Consequently, the pathogens represented herein (E. coli, Salmonella and Staphylococci), were limited to those considered as normal flora in the gut of these animals and/ or those excreted due to bacterial infections. These isolates were further tested for their antimicrobial resistance.

A potential risk arising from the disposal of cow manure is the spread of enteric pathogens.[20] Animals from which food is derived are recognized as reservoirs of many significant food-borne pathogens, including Escherichia coli O157:H7, Salmonella spp., Staphylococci and Campylobacter spp.,[21,22] Many outbreaks or cases of E. coli O157:H7 infection have been associated with water or food directly or indirectly contaminated with animal manure.[23-26] For example, an outbreak of E. coli O157: H7 infection among members of four families was associated with food fertilized with cow manure on the farm.[25] In another instance, a woman acquired E. coli O157: H7 infection associated with eating inadequately washed vegetables that were obtained from a garden fertilized with bovine manure.[26]

There are evidences that imprudent use of antibiotics (antibiotic residues and antibiotic resistance) can adversely affect microbial processes in the environment (e.g. nutrient cycling and pollutant degradation).[15,27,28] Antibiotics administrated to animals provide selective advantages for antibiotic resistant bacteria to develop in animal intestines, which end up in the manures and eventually in the environment. These evidences are entirely consistent with the results of our study that has detected antibiotic-resistant bacteria in dairy cow manure. For instance all the isolates in this study demonstrated resistance to macrolide (Erythromycin, Azithromycin) and Tetracycline. However, Ciprofloxacin resistant was not high as that for macrolides and tetracycline. Moreover, the questionnaire showed that the majority of the visited farms used Tetracycline and macrolides as common antibiotics in these farms and these antibiotics were administered by the owners who lack knowledge about the dose, frequency of the dose and the drug of choice for each disease. Also the treated cows were not kept in separate places and their remnants (mastitic milk, excretions, antibiotics containers) were not removed and find their way directly to the manure. All these factors were considered as means that instigate antibiotic resistant bacteria in manure.

In the United States, almost 80% of the total antibiotics sold are used in the livestock industry and that 40–95% of the administered antibiotic is excreted in faeces and urine where there is the potential to markedly increase antibiotic resistance in soil microbial communities.[29-32] Compounding this probability is the observation that manure from cattle not administered antibiotics can also

stimulate an increase in antibiotic resistance in the microbial community.[31]

5. CONCLUSION

Manure contains abundance of viable antibiotic-resistant bacteria; also it increased the frequency of detection of some resistant gene targets. We attributed the existence of the resistant bacteria in the dairy farms manure to the imprudent usage of antibiotics in these farms. Livestock farms generate huge quantities of animal manure, which must be properly handled, managed and treated. Thus, further work is required to determine the efficacy of manure treatment practices (composting, anaerobic digestion, lime stabilization) in eliminating antibiotic resistance determinants of concern from manures.

ACKNOWLEDGEMENT

Authors would like to thank the staff members of College of Veterinary Medicine, University of Bahri, Sudan for their cooperation and support.

Competing Interest
The authors declare that they have no competing interests.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors**.**

REFERENCES

1. Yang F, Li R, Cui Y, Duan Y-H. Utilization and develop strategy of organic fertilizer resources in China. Soil and Fertilizer Sciences in China, 2010; 4: 77–82.
2. Wang L, Mankin KR, Marchin GL. Survival of fecal bacteria in dairy cow manure.Trans. Am. Soc. Agric. Eng, 2004; 47: 1239–1246.
3. Wang F, Ma W, Dou Z, Ma L, Liu X,Xu J, et al. The estimation of the production amount of animal manure and its environmental effect in China. China Environmental Science, 2006; 26: 614–617.
4. Pachepsky YA, Sadeghi AM, Bradford SA, Shelton DR, Guber AK, Dao T. Transport and fate of manure-based pathogens: Modeling perspective. Agric. Water Manag, 2006; 86: 81–92.
5. Doyle M and Erickson MC. Reducing the carriage of food borne pathogens in livestock and poultry.Poult.Sci, 2006; 85: 960–973.
6. Wichmann F, Udikovic-Kolic N, Andrew S, Handelsman J. Diverse Antibiotic Resistance Genes in Dairy Cow Manure.*mBio*, 2014; 5(2): 01017-13. DOI: 10.1128/ mBio. 01017-13.
7. Yost CK, Diarra MS, Topp E. Animals and humans as sources of fecal indicator bacteria, p 67–92. In Sadowsky MJ, Whitman RL (ed), The fecal bacteria. ASM Press, Washington, DC., 2011.
8. Jechalke S, Kopmann C, Rosendahl I, Groeneweg J, Weichelt V, Krögerrecklenfort E, Brandes N, Nordwig M, Ding G-C, Siemens J, Heuer H, Smalla K. Increased abundance and transferability of resistancegenes after field application of manure from sulfadiazine-treated pigs. Appl. Environ. Microbiol., 2013; 79: 1704 –1711.
9. Gaze WH, Zhang L, Abdouslam NA, Hawkey PM, Calvo-Bado L et al. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. ISME J., 2011; 5: 1253–1261.
10. Heuer H, Schmitt H, Smalla K. Antibiotic resistance gene spreaddue to manure application on agricultural fields. Curr. Opin. Microbiol., 2011; 14: 236 –243.
11. Wellington EMH, Boxall ABA, Cross P, Feil EJ, Gaze WH, HawkeyPM, Johnson Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM,Williams AP. The role of the natural environment in the emergenceof antibiotic resistance in Gram-negative bacteria. Lancet Infect. Dis., 2013; 13: 155–165.
12. Barrow G I and Feltham RKA. Cowan and steel's manual for the identification of medical bacteria. 3rd edition, Cambridge University Press, 1993.
13. CLSI, Clinical and Laboratory Standards Institute–approved standards: M02-A12—Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Twelfth Edition, 2016; M07-A10.
14. Wepking C, Avera B, Badgley B, Barrett JE, Franklin J, Knowlton KF, Ray PP, Smitherman C, Strickland MS. Exposure to dairy manure leads to greater antibiotic resistance and increased mass-specific respiration in soil microbial communities. Proc. R. Soc. B., 2017; 284: 20162233. http://dx.doi.org/10.1098/rspb.2016.2233.
15. Sarmah AK, Meyer MT, Boxall AB. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere, 2006; 65: 725–759.
16. Pol M and Ruegg PL. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J. Dairy Sci., 2007; 90: 249-261.
17. Almofti YA, Elnasri HA, Salman AM, Ashri FO. Imprudent usage of antibiotics in dairy farms and antibiotics detection in milk. Ann Biolog Res, 2016; 7(5): 36-42. (http://scholarsresearchlibrary.com/archive.html).
18. Eltayb A, Barakat S, Marrone G, Shaddad S, LundborgSC. Antibiotic use and resistance in animal farming: a quantitative and qualitative study on knowledge and practices among farmers in Khartoum, Sudan. Zoonoses Public Health., 2012; 59(5): 330-8.
19. Bagge E. Hygiene Aspects of the Biogas Process with Emphasis on Spore Forming Bacteria. Ph.D. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden, 2009.

20. Braykov NP, Eisenberg JNS, Grossman M, Zhang L, Vasco K et al. Antibiotic resistance in animal and environmental samples associated with small-scale poultry farming in North western Ecuador. M Sphere, 2016; 1: 21 -15.
21. Doyle M P, Zhao T, Meng J, S Zhao S. Escherichia coli O157:H7, p. 171–191. In M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers.ASM Press, Washington, D.C., 1997.
22. Elder R O, J E, Keen G R, Siragusa G A,Barkocy-Gallagher, M. Koohmaraie, and W WLaegreid. Correlation of enterohemorrhagic Escherichia coli O157:H7 prevalence in feces, hides and carcasses of beef cattle during processing. Proc. Natl. Acad. Sci. USA, 2000; 97: 2999–3003.
23. Centers for Disease Control and Prevention (CDC). Outbreaks of Escherichia coli O157:H7 infections among children associated with farm visits-Pennsylvania and Washington, 2000. Morb. Mortal. Wkly. Rep., 2001; 50: 293–297.
24. Centers for Disease Control and Prevention (CDC). Outbreak of Escherichia coli O157:H7 infections associated with drinking unpasteurized commercial apple juice—British Columbia, California, Colorado, and Washington, October 1996. Morb.Mortal. Wkly. Rep., 1996; 45: 975.
25. Chapman PA, Siddons CA, Manning J, Cheetham C. An outbreak of infection due to verocytotoxin-producing Escherichia coli O157 in four families: the influence of laboratory methods on the outcome of the investigation. Epidemiol. Infect, 1997; 119: 245–250.
26. Cieslak PR, Barrett TJ, Griffin PM, Gensheimer KF, Beckett G, Buffington J, Smith MG. Escherichia coli O157:H7 infection from amanured garden. Lancet, 1993; 342: 367.
27. Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K. Fateand effects of veterinary antibiotics in soil. Trends in Microbiology, 2014; 22: 536–545.
28. Larson C. China's lakes of pig manure spawn antibiotic resistance. Science, 2015; 347: 704.
29. Gutierrez IR, Watanabe N, Harter T, Glaser B, Radke M. Effect of sulfonamide antibiotics on microbial diversity and activity in a Californian Mollic Haplo xeralf. J. Soils Sediments, 2010; 10: 537–544. (doi:10.1007/s11368-009-0168-8).
30. Kemper N. Veterinary antibiotics in the aquatic and terrestrial environment. Ecol. Indic., 2008; 8: 1–13. (doi:10.1016/j.ecolind.2007.06.002).
31. Udikovic-Kolic N, Wichmann F, Broderick NA, Handelsman J. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc. Natl Acad. Sci. USA, 2014; 111(15): 202–15-207. (doi:10.1073/pnas.1409836111)
32. Toth JD, Feng YC, Dou ZX. Veterinary antibiotics at environmentally relevant concentrations inhibit soil iron reduction and nitrification. Soil Biol. Biochem, 2011; 43: 2470–2472. (doi:10.1016 /j.soilbio. 2011.09.004).