Pharmaceutical Journal of Sri Lanka 2017 *Volume 7, Issue 1*
http://doi.org/10.4038/pjsl.v7i0.16

Research Article

Risk of Prophylactic Antibiotics in Livestock and Poultry Farms; A Growing Problem for Human and Animal Health

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Abstract

Purpose: Veterinary antibiotics are physiologically highly active substances which are being used on a large scale as therapeutic drugs and as feed additives in modern agricultural practice. Major purposes of use of antibiotics are prevention and treatments of bacterial infections and for the improvements of growth rates of farm animals. Therefore, the present study was focused on quantification of some selected groups of antibiotics; tetracycline [Tetracycline (TET), Oxytetracycline (OTC)] and penicillin [Amoxicillin (AMX), Ampicillin (AMP)] in wastewater discharge drains in large-scale livestock and poultry farms and to isolate antibiotic resistant bacteria.

Methods: Twenty wastewater discharge drains in livestock and poultry farms were selected to collect samples for the study. Solid-Phase Extraction (SPE) was employed and antibiotic quantification was done by High Performance Liquid Chromatography (HPLC). Isolation of resistance (r) bacteria was done by standard pour plate method where Minimum Inhibitory Concentration (MIC) of bacteria was determined using the range of 60 to 720ppm concentrations of the antibiotics by agar dilution method.

Results: Oxytetracycline (55%), and tetracycline (38%) were recorded as most frequent antibiotics in samples and having concentrations of 0.005 ppm and 0.004 ppm respectively. The highest concentration of AMX was recorded as 0.003±0.004 ppm. However, AMP was not detected during the study period. Oxytetracycline and tetracycline resistance bacteria were identified as *Enterococcus* sp., *E. faecium, E.coli* and *Clostridium* sp. while, *A. baumanii, E. clocae, A. lwoffii* and *H.pylori* were identified as AMPr and AMXr bacteria by 16S rRNA sequencing. The MIC values of tetracycline (TET, OTC) resistant bacteria ranged from 360 ppm to 720 ppm whereas for penicillin (AMX, AMP) from 360 to 760 ppm.

Conclusion: The contamination of antibiotics leads to develop antibiotic resistance in environmental bacteria. Thus, the results of the study indicate that presence of antibiotic resistant bacteria may limit the effectiveness of antibiotics in treating animal illness, thereby causing a potential risk to the productivity of livestock and poultry farms.

Keywords: Tetracycline, Oxytetracycline, Amoxicillin, Ampicillin, Resistance

Introduction

Antibiotics are the most important group of antimicrobial drugs, widely prescribed for human, and animal diseases. According to the World Health Organization (WHO), about half of the antibiotics are consumed for non-human applications.(1,2) Livestock farming is frequently referred to as a reservoir for potentially pathogenic and antimicrobial resistant bacteria and as a reservoir of antibiotic resistance genes as well.(3,4) Antimicrobial drugs are widely applied in animal husbandry to increase production, treatment of infectious diseases and as growth promoters.(5)

Tetracycline, β-lactams and macrolides are the most common antibiotic groups which are being used for veterinary purposes, human therapy and in agriculture sector as feed additives.(6) Owing to their lower cost and their higher antimicrobial activity, tetracycline antibiotics are widely used as veterinary drugs for the prevention and treatment of several infectious diseases such as leptospirosis, mycoplasma.(7) Tetracycline antibiotics are used as feed additives to promote the growth of the animals.(8) Several β-lactams are commonly used for infections such as; colibacillosis, fowl cholera, respiratory infections due to *Ornithobacterium rhinotracheale*, septicemia due to *Riemerella antipestifer* (ampicillin, amoxicillin), dysbacteriosis (benzyl penicillin), and erysipelas (penicillin) in poultry.(3, 4)

According to the available literature (4, 9) antibiotics can enter into the aquatic environment via the direct discharge of animal wastewater and effluent water from wastewater treatment. Besides, the application of manure from livestock farm onto agriculture land contributes to introduce antibiotics in to the aquatic environment.(3,4) The introduction of these residual compounds into the environment through different sources will lead to serious environmental problems including environmental pollution and risk of wide spread transmission of antibiotic resistant pathogenic bacteria to humans.(10) Afterwards, transfer of resistance genes from antibiotic resistance microorganisms to indigenous environmental bacteria occurs under selection pressure of antibiotics driven by the mechanism of horizontal gene transfer (HGT).(11) In soil, transfer of resistance determinants to pathogens may reduce the efficiency of antibiotics on both human and animals. (12,13) Thus recently, antibiotic resistance genes (ARG) and antibiotic resistance bacteria were considered as new emerging contaminants.(10,14)

At present, large amounts of antibiotics are used to intensively manage animal husbandry in Sri Lanka. This has resulted in high contamination levels of veterinary antibiotics in wastewater from livestock farms.(5) Thus, the present study was planned to determine antibiotic contamination status and screening of antibiotic resistance bacteria in effluent waters in livestock farms in Sri Lanka.

Methods

Standards and reagents

Two tetracyclines including oxytetracycline (OTC) (97%), tetracyclines (TET) (97%),

two penicillin including amoxicillin (AMX) (96%), ampicilline (AMP) (97%) and all the HPLC grade chemicals were purchased from Sigma Aldrich, USA.

Sample collection

Triplicate effluent water samples were collected from 20 sampling locations: including 10 livestock farms (Ambewala - New Zealand, Ambewala – Highland, Bopaththalawa, Weerawila, Jaffna, Batticaloa, Dayagama, Ridiyagama, Nikwaratiya) and 10 poultry farms (Karandagolla, Katunayake, Jaffna, Bairaha, Gampaha) following standard methods (Figure 1).(15)

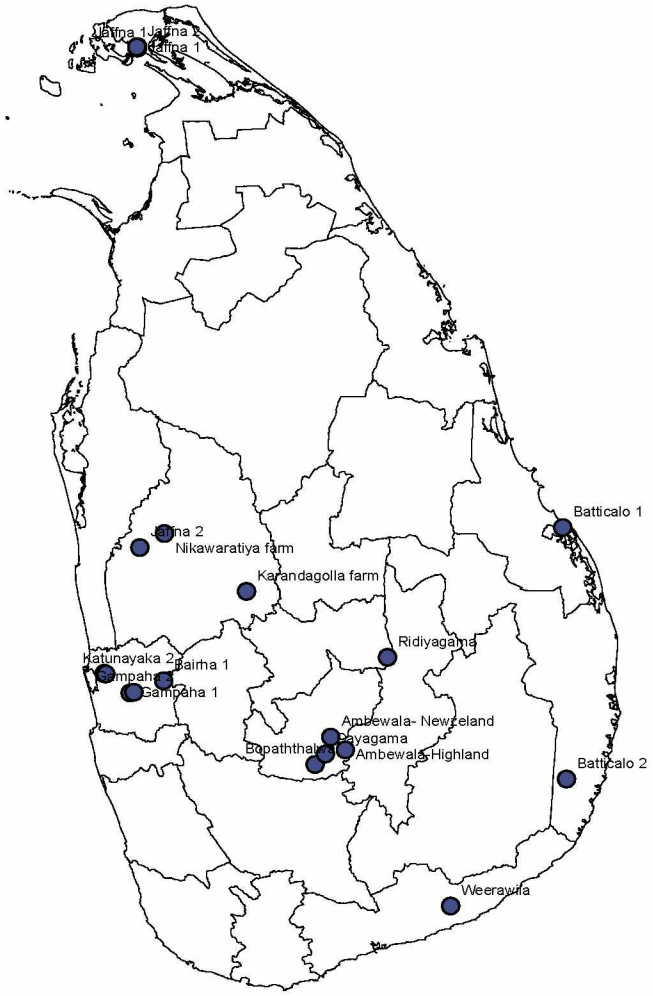

Figure1: Effluent water sampling locations of the present study

Antibiotic analysis

One liter of wastewater sample was adjusted to pH 3 and the sample was filtered through 0.22 µm nuclearpore filter. Samples were spiked with antibiotic at final concentration of 100 ppm and subjected to SPE.(16,17) In this procedure, C18 cartridge was preconditioned with 5 ml 100% methanol and then 5ml double-deionized water. Pre-prepared samples were then passed through

the C18 cartridges at flow rate of approximately 1-2 ml/min and then rinsed with 5 ml of deionized water. The analytes were eluted with 5ml of 100% methanol followed by 2ml of 100% isopropanol. The extract was purged by 99.9% nitrogen stream and re-dissolved with 1ml of mobile phase.(18,19) The target antibiotics were quantified by using Agilent 1200 series HPLC equipped with diode array and fluorescence detector. Antibiotics in effluent water were analyzed according to the Fernandez et al. (2010) with a modification.(20) 0.1% glacial acetic acid (polar protic solvent) was used as mobile phase and injected volume was 20 µl and chromatography was performed at 30 ^{0}C. The mobile phase of the mixture of 0.1% glacial acetic acid in water (Component A): 0.1% glacial acetic acid in acetonitrile (Component B), 99:1 (v/v) was pumped beginning at a flow rate of 0.7 ml/min for OTC, TC, AMP and AMX. Then followed linear elution gradient from 99% to 70% A in 25 min for antibiotics.(15,17,19) Table 1 shows conditions and retention time employed for each antibiotic analysis.

Table 1: Wave length and retention time for different antibiotic tested in the present study

Analyte	λ absorption (nm)	Retention time (min)	SD (min) (n=3)
OTC	280	17.34	0.002
TET	280	16.56	0.001
AMP	230	13.08	0.001
AMX	230	11.23	0.006

SD, Standard deviation

Calibration plots

Individual calibration standards of each antibiotic (OTC, TET, AMP, AMX) at 0-100 ppm were prepared. The linearity of the method was evaluated by plotting peak area as a function of the concentration of each analyte.

Enrichment and isolation of antibiotic resistance bacteria

50 ml of water from each sampling sites were enriched by spiking, antibiotic at final concentration of 60 ppm in 100 ml erlenmeyer flasks and the final volume was topped up to 100 ml with sterile water and then the flasks were incubated at 28 ^{0}C ± 1 ^{0}C in 100 rpm for 14 days in a shaking incubator. After 14 days of incubation, 1 ml of sample aliquot was taken from each flask. Isolation and enumeration of bacteria was carried out by standard pour plate method.(10) LB medium containing 60 ppm of antibiotics (TET, OTC, AMP, AMX) was used to isolate TET, OTC, AMP and AMX resistant (r) bacteria.(15,17,21)

After three days of incubation at 28 ^{0}C, bacteria colonies with different morphological characters were picked up and re-suspended in sterilized liquid LB medium. Subsequently pure bacterial cultures were sub cultured and stored in agar slants at -20 ^{0}C in LB-glycerol media for further studies and identification purposes.(22)

Antibiotic susceptibility test

The LB broth culture was prepared and a loop of isolated bacteria strain was inoculated and incubated at 28 ^{0}C shaking under 100 rpm overnight. The cell density of the bacteria suspension was equalized using McFarland No 0.5.(15,21) MIC was determined by using an agar dilution method following the CLSI guidelines.(23)

Results and Discussion

Antibiotics are commonly used at sub therapeutic levels in livestock to prevent diseases and promote growth.(24) Despite the long history of antibiotic usage, information regarding antibiotic production and usage patterns in Sri Lanka is severely limited due to the lack of research information, comprehensive monitoring and documenting efforts. The large amounts of antibiotic usage may result in their presence in environment as up to 90 percent of the administered antibiotics are excreted without undergoing metabolism.(25)

Based upon the WHO estimates, considerable differences were recorded in antibiotic usage among different animal species (swine vs. poultry). Therefore, the types of antibiotic compounds that are likely to be found in effluent water of livestock farms will strongly depend upon the types of livestock operations within the environment.(7)

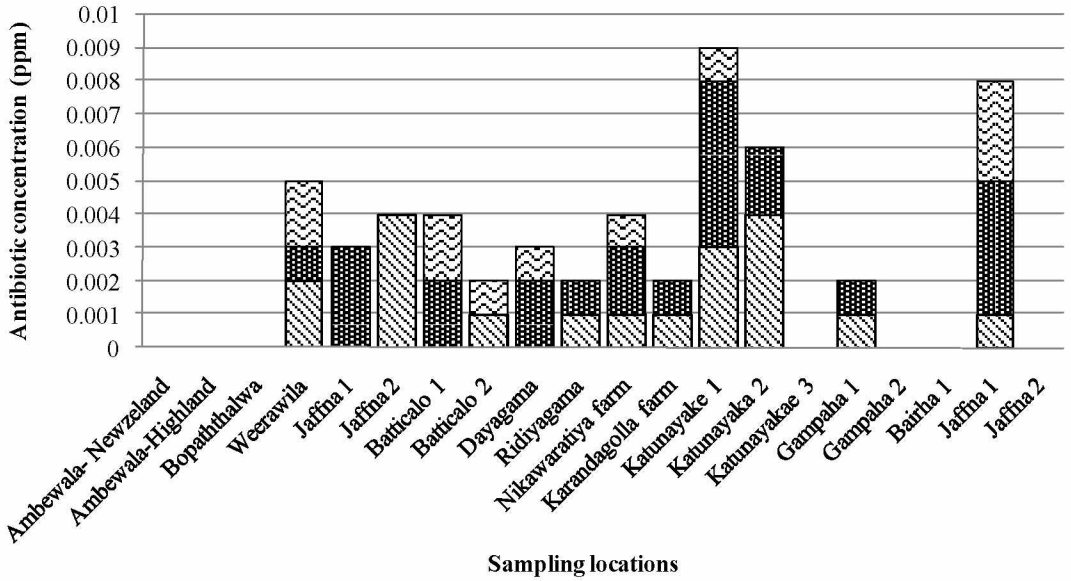

Figure 2: Antibiotic contamination levels in livestock farms (n=3); ▨ OTC, ▩ TET, ▨ AMX, ■ AMP

In the present study antibiotic concentrations in effluent water of livestock farms ranged between 0.001 ppm-0.004 ppm for OTC, 0.001 ppm - 0.005 ppm for TET, 0.001 ppm-0.003 ppm for AMX respectively (Figure 2). However, AMP was not detected during the study period. The maximum concentrations of AMX (0.003 ppm) and OTC (0.004 ppm) were detected in samples collected from livestock farms in Jaffna (Figure 2). The considerable concentration of TET (0.005± 0.021 ppm) was detected in effluent water at a poultry farm in Katunayake.

Among the selected antibiotics, approximately 45% from the TET, 35% from the OTC and 20% of AMX contributed to environment pollution through the effluent water.

The WHO has recommended less than 0.001 ppm as guideline value for antibiotic

residues in the aquatic environment. (26,27,28) Thus, the results of the present study revealed that the antibiotic contamination level in effluent water had exceeded the values given by the WHO.(26)

The high detection frequency and concentration of tetracyclines are likely due to the large amount of OTC and TET usage in livestock and poultry as feed additives and therapeutic drugs for diseases in farm sites. The results of the present study are comparable to the findings of Brown et al., (2010), who reported concentrations of OTC (0.001-0.008 ppm), TET (0.001-0.005 ppm), AMX (not detected) and AMP (not detected) in livestock effluents in New Mexico. Similar to previous studies(21,29) conducted in different parts of the world, in the present study too, effluent water of livestock farms were recorded as a significant source of antibiotic contamination. In the present study OTC (0.001-0.004 ppm) and TET (0.001-0.006 ppm) were detected at high concentrations while AMX (0.001-0.00 2ppm) was recorded in low concentrations.

These antibiotic residues promote the evolution or the development of antibiotic resistant microorganisms, which can induce adverse effect to human health when present in drinking water or irrigation water.(21,29) These antibiotic resistant bacteria may disturb the microflora of the human intestines and increase the risk of certain infectious diseases as well.(3,26)

Figure 3: Phylogenetic affiliations for OTCr, TETr, AMXr and AMPr bacteria with their phylogeny and Minimum Inhibition Concentration (MIC) values, The phylogeny was constructed by neighbor-joining method based on Mega 6/ Cluster W from alignment of 16s rRNA gene sequence comparison of antibiotic resistance bacteria.

Name of isolate	Accession No	MIC (ppm)
Bacillus subtilis (AMX 4)	HF 586509	480
Bacillus sp. (TET 3)	HF586507	640
Bacillus sp. (TET 4)	HF657890	720
Bacillus sp. (AMP 4)	KU567678	360
Bacillus thurengiensis (AMP 3)	NR.042387	400
Enterobacter sp. (OTC 5)	EU.159482	420
Enterobacter cloacae (AMX 7)	KL 234156	680
Enterobacter cloacae (AMX 1)	KM 435657	360
Escherichia coli (AMX 3)	KN 514297	700
Escherichia coli (AMX 11)	FJ 982654	480
Escherichia coli (TET 2)	FJ 922659	660
Escherichia coli (AMP 1)	FJ 982657	720
Escherichia coli (OTC 3)	D1186353	360
Enterobacter faecium (TET 1)	KM234561	380
Enterobacter cloacae (AMX 5)	KX 495499	400
Streptococcus equi (AMX 9)	HF 947328	540
Lactobacillus sp. (AMP 7)	EU 321541	400
Enterococcus sp. (OTC 2)	AY 499112	360
Enterococcus sp. (TET 4)	KX 458221	480
Helicobacter pylori (AMP 80	FS 464023	380
Citrobacter freundii (AMX 2)	EU 321541	680
Citrobacter freundii (AMX 8)	AY 499112	720
Acinetobacter baumanii (AMP 2)	KX 458221	360
Acinetobacter lwoffii (AMP 6)	FS 464023	580
Streptomyces rimosus (AMX 5)	KU 690365	620
Clostridium perfringens (AMX 10)	KU 691844	360
Clostridium sp. (OTC 1)	KS 678976	380
Streptomyces sp. (AMX 6)	FJ 982654	460

In the present study different antibiotic resistance bacteria were isolated based on MIC test. The isolates were subjected to the 16S rRNA analysis to identify the isolated OTCr, TETr, AMPr and AMXr. Species composition results for OTC, TET, AMP, and AMX resistant bacteria in environmental samples from different sampling sites and their MIC values are summarized in Figure 3.

Based on the 16S rRNA sequences, most of the resistance bacteria belonged to genera of *Bacillus*, *Enterobacter* and *Escherichia*. The majority of tetracycline resistant bacteria belonged to the genera *Bacillus*, *Enterococcus, Enterobacter, Clostridium, Escherichia* whereas penicillin (AMX, AMP) resistant bacteria belonged to *Escherichia, Enterobacter, Streptomyces, Streptococcus, Lactobacillus, Bacillus, Helicobacter, Acinetobacter* and *Clostridium*.

The MIC values of OTCr bacteria in livestock farms ranged between 360 ppm to 720 ppm whereas for TETr bacteria were from 360 to 700 ppm (Figure 2). MIC values of AMXr bacteria ranged between 360- 720 ppm; the highest MIC was recorded for *Citrobacter freundii* where the lowest was for *Bacillus* sp. MIC of AMPr bacteria varied from 360 ppm to 720 ppm whereas the highest MIC was recorded for *E.coli* and the lowest for *Bacillus* sp. respectively.

The results of the present study were different from the study conducted in Vietnam, where *Acinetobacter* was the most common tetracycline resistant bacterium.(5) However, another study in a Vietnamese poultry farm found that *Vibrio* sp. was the commonest tetracycline-resistant bacteria, followed by *Bacillus* sp.(4) These results may reflect regional differences in species composition of the bacterial population.

In the present study, most of OTCr and TETr bacteria were isolated from livestock farms and those bacteria mainly caused nonscomial infections (*Enterococcus* sp., *E. faecium, Enterococcus* sp.) and intestinal infectious diseases (*E.coli*, *Clostridium* sp.). However, *Enterobacter* sp. and *Bacillus* sp. were recorded as non pathogenic bacteria. Most AMXr and AMPr bacteria which were isolated from livestock farms were recorded as causative agents for intestinal diseases and opportunistic infections, whereas only few non-pathogenic bacteria were detected. (8,29)

There is a further concern that antibiotic-resistant bacteria might develop from long-term environmental exposure to low concentrations of antibiotic (ng/L-µg/L), present in wastewater and surface waters. (30) It was highlighted that continuous exposure to a particular antibiotic residues in the environment, multidrug resistant pathogens can make drugs ineffective and pose a serious risk to the global pharmaceutical and healthcare industry.(10, 31)

Thus, greater attention must be given to the overall pattern of antibiotics usage in the farms, including environmental risk assessment and research on the mitigation of antibiotic contaminants. Therefore the baseline data collected in the present study will provide first-hand information to authorities to open eyes to see the contamination status and develop national strategic plan to reduce antibiotic contamination from anthropological

activities in order to safe guard human and animal health.

Conclusion

In conclusion, the present study demonstrates that livestock and poultry farms have been a reservoir of antibiotics and antibiotic-resistant bacteria. The TETr, OTCr, AMPr and AMXr bacteria; especially the opportunistic pathogens were isolated from farm wastewater and the contaminations of TET, OTC, AMP and AMX implies an urgent need for constructing a monitoring system for antibiotic usage in livestock and poultry farms. Because isolated antibiotic resistance bacteria in farm wastewater may lead to problems in the efficiency of antibiotics which used to treat animal diseases. Further research is particularly necessary to better understand the route and mechanisms of ARGs proliferation and to thus mitigate the risks to public health.

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