P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article

Molecular Detection of Tetracycline and Sulfonamide Resistance Genes in Respiratory and Gastrointestinal Bacterial Isolates of Ruminants

Gemerlyn G Garcia[1§], Allan Jeffrey E Francia[1], Kevin B Costales[1], Michelle M Balbin[3] and Claro N Mingala[2,3§]

[1]College of Veterinary Medicine, Central Luzon State University, Science City of Muñoz 3120, Nueva Ecija, Philippines
[2]Department of Animal Science, College of Agriculture, Central Luzon State University, Science City of Muñoz 3120, Nueva Ecija, Philippines; [3]Philippine Carabao Center National Headquarters and Gene Pool, Science City of Muñoz 3120, Nueva Ecija, Philippines
[§]These authors contributed equally to this work.
***Corresponding author:** cnmingala@hotmail.com

Article History: Received: September 03, 2018 Revised: November 15, 2018 Accepted: December 08, 2018

ABSTRACT

The resistance of respiratory bacterial isolates (*Acinetobater schindleri, Bacillus pumilus, Enterococcus faecalis, Pseudomonas aeruginosa, Staphylococcus sciuri and Staphylococcus sporosarcina*) and gastrointestinal isolates (*Arthrobacter sp., Bacillus megaterium, Pseudomonas aeruginosa, Escherichia coli and Enterococcus faecalis*) from ruminants to tetracycline and sulfonamide was evaluated. Antibiotic sensitivity test in agar cup well method that applied different concentrations of tetracycline and sulfonamide demonstrated the resistance of the isolates to different concentrations of tetracycline and sulfonamide. Tetracycline and sulfonamide resistance in antibiotic sensitivity test was further validated by PCR amplification of genes that code for tetracycline and sulfonamide resistance. Methods involved the utilization of primers that recognize efflux pumps (*tet*B), ribosomal protective proteins (*tet*M) and enzyme inactivation (*tet*X) in genes that regulate tetracycline resistance in bacteria while testing for sulfonamide resistance involved the application of primers for dihydropteroate synthase (DHPS) genes (*sul1* and *sul2*). DNA sequencing of amplified products revealed tetracycline resistance in one respiratory bacterial isolate *(E. faecalis*) out of the 6 isolates tested. The amplicon with the putative tetracycline resistance had a molecular weight of 171 bp and explains the involvement of ribosomal protective proteins encrypted in the *tet*M gene as a mediator of tetracycline resistance in *E. faecalis*. Sulfonamide resistance gene was exhibited by the GIT bacterial isolate *P. aeruginosa.* The DNA amplicon with the reputed sulfonamide resistance is linked with the *sul2* genes which had a molecular weight of 721 bp. The detection of the *sul2* genes explains the inhibition of DHPS to effect resistance of *P. aeruginosa* to sulfonamides.

Key words: Antibiotic sensitivity test, *E. faecalis*, Polymerase chain reaction, *Pseudomonas aeruginosa*, *sul*2 gene, *tet*M gene, Sulphonamide resistance genes, Tetracycline resistance gene

INTRODUCTION

Small and large ruminants reared in commercial and backyard conditions commonly succumb to respiratory and gastrointestinal infections especially at the onset of the rainy season. Previous study cited the isolation and identification of bacteria like *Acinetobacter schindleri (A. schindleri), Staphylococcus sciuri (S. sciuri), Staphylococcus sporosarcina (S. sporosarcina), Pseudomonas aeruginosa (P. aeruginosa), Bacillus pumilus (B. pumilus)* in respiratory and *Enterococcus faecalis (E. faecalis)* in gastrointestinal infections *(*Garcia *et al., 2013).* This necessitated continuous evaluation of the responses of these bacteria to treatment (Garcia *et al.*, 2015) which surprisingly revealed their resistance to antibiotics.

Tetracyclines are broad-spectrum antimicrobial agents with defined functions in altering protein synthesis in a wide range of Gram-positive and Gram-negative bacteria, chlamydiae, mycoplasma, rickettsiae and protozoan parasites (Kordick *et al.*, 1997). The antimicrobial properties and the absence of major adverse side effects in tetracycline have led to the extensiveness of their use in the therapy of human and animal infections (Levy, 1992) which gradually contributed to the reported emergence of tetracycline resistance and posed drawbacks in its therapeutic value (Chopra and Roberts, 2001).

Cite This Article as: Garcia GG, AJE Francia, KB Costales, MM Balbin and CN Mingala, 2019. Molecular detection of tetracycline and sulfonamide resistance genes in respiratory and gastrointestinal bacterial isolates of ruminants. Inter J Vet Sci, 8(1): 1-9. www.ijvets.com

Sulfonamides are reportedly the oldest but remain among the most widely used antibacterial agents in veterinary medicine, chiefly because of low cost and their relative efficacy in common bacterial diseases (Messi *et al*., 2005). These drugs are administered in feeds, in water and by injection to treat conditions of pneumonia, scours, coccidiosis, foot rot, calf diphtheria, acute mastitis and metritis (Lanz *et al*., 2003).

A significant increase in the prevalence of antimicrobial resistance has been recognized as an emerging problem in veterinary therapeutics. Infection by antimicrobial resistant bacteria may not only curtail an efficient treatment but may intensify morbidity and mortality (Collignon *et al*., 2016). Repeated use of antibiotics that reportedly induces drug resistance while the transfer of resistance genes from one bacterium to another cater their entry to the food web and further add to public health concerns (Reynolds *et al*., 2003).

In light with reports on the emergence of antibiotic resistance in bacteria, effort was extended toward the evaluation of bacteria associated with respiratory and gastro-intestinal infections of ruminants and their responses to treatment with tetracycline and sulfonamide while paying special attention on the possible detection for resistance on these 2 antibiotics. In the deficiency of information on the extent of antimicrobial resistance in animal practice, animals harbouring tetracycline and sulfonamide resistance can be a potential food-borne vehicle for resistance genes for both animal and human disease pathogens. Assessment of antimicrobial resistance at molecular level is a useful tool for understanding the role of genetic elements for the development and dissemination of resistance in bacteria (Alekshun and Levy, 2007). A study was then initiated to investigate and validate genes responsible in mediating tetracycline and sulfonamide resistance. Data generated from this research work provide reference in defining alternative and other rational lines of treatment. This may also help farmers, livestock practitioners and veterinarians in disregarding the practice on empirical antimicrobial therapy that may cater drug resistance.

MATERIALS AND METHODS

Bacterial strains

Pure culture of six bacterial isolates previously identified by DNA sequencing (*A. schindleri*, *B. pumilus*, *E. faecalis*, *P. aeruginosa*, *S. sciuri* and *S. sporosarcina*) (Garcia *et. al.*, 2013) from respiratory-ill ruminants that received tetracycline and GIT bacterial isolates, *Arthrobacter* sp., *Bacillus megaterium (B. megaterium)*, *P. aeruginosa*, *Escherichia coli (E. coli)* and *E. faecalis*, from diarrheic animals which received sulphonamides were used in the study. A record of the antibiotics commonly used in ruminant infections during the period of study was obtained (Table 1).

In-vitro screening for the sensitivity of bacterial isolates to tetracycline and sulphonamide

Commercial preparations of tetracycline and sulfonamide were prepared in different concentrations (50 µg/ml, 25 µg/ml, 10 µg/ml, 5 µg/ml and 0 µg/ml). Bacterial suspensions (500 µl) were adjusted separately at 0.5 MacFarland then added to Mueller Hinton Agar before overlaying on five replicated plates. Five holes each with a diameter of 7.9 mm, depth of 6.0 mm to accommodate a volume of 50 µL were made for the indicated concentrations of tetracycline. All plates were incubated at 37^{o}C for 24 hrs and the diameters of the zones of inhibition were measured after incubation. All data were taken and computed as means of five replicates (± Standard deviation). Breakpoints for susceptibility (≤4 µg/ml) and resistance (≥16 µg/ml) to tetracycline; and susceptibility (≤256 µg/ml) and resistance (≥512 µg/ml) to sulphonamide were adapted (Clinical Laboratory Standards Institute/CLSI M100, 28th Ed, 2018). The breakpoints given only exist for *S. aureus. Enterococcus fecalis and E. coli* and were taken as reference. No CLSI interpretation criteria exists for the other bacteria and antibiotics are currently available (https://www. fda.gov/).

DNA extraction

Each bacterial isolate (18-hr old culture) was transferred aseptically in tubes that contained 37 µL TE buffer. DNA extraction of each sample was undertaken following the manufacturer's protocol (Promega, USA). Briefly, overnight cultures were centrifuged at 14,000 x g for 2 min and suspended in 4.8 mL 50 mM EDTA. The cell lysis solution is added (120 µL) before the samples were heated at 37°C for 30 to 60 min. After heating, the samples (suspension) were centrifuged at 14,000 x g for 2 min before discarding the supernatants are discarded. The nuclei lysis solution (600 µL) was added to the pellets before incubation at 80°C for 5 min. The samples were allowed to cool at RT before adding the RNA solution. The samples were incubated at 37°C for 30 to 60 min before adding the protein precipitating solution (200 µL per sample). The samples were incubated in ice for 5 min before centrifugation at 14,000 x g for 3 min. The supernatant samples were collected and dispensed in sterile tubes before the addition of isopropanol (500 µL). The samples were centrifuged at 14,000 x g for 2 min. The supernatants were discarded before the addition of Ethanol (70%, 600 µL) to the pellets. The samples were

Table 1: Commonly used antibiotics in ruminant infections during the period of study

Antibiotics	Reason for antibiotic administration	Animal species	No. of sick animals that received treatment
Oxytetracycline, Enrofloxacin, Penstreptomycin	Respiratory infections	Water buffaloes	5
Tylosin, Sulfonamide	Diarrhea		2
Oxytetracycline, Enrofloxacin, Penstreptomycin	Respiratory infections	Cattle	5
Tylosin, Sulfonamide	Diarrhea		3
Oxytetracycline, Penstreptomycin	Respiratory infections	Goats	10
Sulfonamide	Diarrhea		10
Oxytetracycline, Penstreptomycin	Respiratory infections	Sheep	5
Sulfonamide	Diarrhea		5

centrifuged again at 14,000 x g for 2 min. The pellets (DNA) were allowed to dry then stored at -20°C until use. The DNA samples were rehydrated (Hydrating solution (50 µL) before PCR.

Primers for PCR

For the evaluation of tetracycline resistance, a pair of *tet*B primers (Forward sequence, 5'-TACGTGAATTTAT TGCTTCGG-3' and Reverse sequence, 5'-ATACAGCA TCCAAAGCGCAC-3') that reportedly (Aminov *et al*., 2002) recognize a 206-bp gene that encodes for tetracycline resistance-mediated efflux pumps were used. Another pair of primers (*tet*M, Forward sequence 5'-ACAGAAAGCTTATTATATAAC-3' and Reverse sequence, 5'-TGGCGTGTCTATGATGTTCAC-3') were utilized to detect a 171 bp gene which targets tetracycline resistance-mediated ribosomal protective proteins as described (Aminov *et al*., 2001). The third set of primer (*tet*X) which were used by others (Ghosh *et al*., 2008) detect tetracycline resistance-mediated enzyme inactivation after the amplification of a target gene with a 1167 bp (Forward sequence 5'-ATGACAATGCGAATA GATACAGACA-3' and Reverse, 5'-CAATTGCTGAA ACGTAAAGTC-3') were also used in the study.

For the evaluation of sulfonamide resistance, primers for *sul1* (Forward primer, 5'-GTGACGGTGTTCGGCA TTCT-3' and Reverse primer, 5'-TCCGAGAAGGTGA TTGCGCT-3') and *sul2* (Forward primer, 5'-CGGCATCGTCAACATAACCT-3'and Reverse primer, 5'-TGTGCGGATGAAGTCAGCTC-3') were used to detect genes reportedly carried by plasmids to encode resistance to sulfonamide. The genes were expected to register products with a molecular weight at 779 base pair (bp) for *sul1* and 721 bp for *sul2* (Kashif *et al*., 2013).

DNA amplification through PCR

The reaction mixture included 11.3 µL sterile DDW, 4 µL 5X PCR buffer, 2 µL 0.1mM MgCl2, 0.5 µL 200 µM DNTP, 0.5 µL 0.1 mM forward primer, 0.5 µL 0.1 mM reverse primer, 0.2 µL Taq (0.05 units/µL) and 2 µL DNA template (100 pg/ µL) making up a total volume of 20 µL.

Amplification of the *tet*B required initial denaturation at 94°C for 5 min and 25 cycles denaturation at 94°C for 5 sec, annealing at 55°C for 30 sec, final extension at 61°C for 7 min and a final hold at 4°C**.** Amplification of the *tet*M genes involved initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, a final extension at 72°C for 7 min and a final hold at 4°C. Amplification of the *tet*X genes applied an initial denaturation at 94°C for 5 min and 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec, final extension at 72°C for 2 min and a final hold at 4°C.

Amplification of the *sul1* gene involved initial denaturation at 95°C for 5 min and 35 cycles of denaturation at 95°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 60 sec, final extension at 72°C for 7 min and final hold at 4°C. For the *sul2* gene, amplification applied an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 46°C for 30 sec and extension at 72°C for 60 sec, final extension at 72°C for 7 min and final hold at 4°C.

A 1Kb+ ladder (5 µL, Invitrogen, USA) was used as a reference for determining the molecular sizes of the genes in the absence of a positive control resistance gene for either tetracycline and sulfonamide. The PCR products (3.0 µL) and the 1Kb+ ladder was allowed to resolve in 2% agarose gel before evaluation of amplified products in a trans-illuminator (Gel Doc, USA).

DNA sequencing of tetracycline and sulphonamide resistance genes

The PCR products were purified before submission of samples for Sanger sequencing in a DNA facility (AsiaGel, Malaysia). The nucleotides associated with tetracycline and sulfonamide resistance gene sequences were aligned manually with an available software (Nucleotide BLAST, NCBI).

RESULTS

Large and small ruminants are prone to infections of the respiratory and gastro-intestinal tracts. In the treatment of respiratory infections, oxytetracycline, enrofloxacin and penstreptomycin are reportedly given while tylosin and sulphonamide are chosen for GIT infections (Table 1). Routine protocols that involved in-vitro screening of the sensitivity of the bacterial isolates revealed resistance of respiratory infection-associated bacteria to tetracycline (Table 2) while diarrhea-associated bacteria to sulfonamide (Table 3).

The resistance of the bacterial isolates to tetracycline and sulfonamide noted *in-vitro* was further validated by the amplification of genes that recognize tetracycline and sulfonamide resistance through PCR.

Table 2: Phenotypic resistance profile of 6 bacterial isolates associated with respiratory infections of ruminants to different concentrations of tetracycline

	Levels of Tetracycline application (µg/mL)					
Bacteria	0	5	10	25	50	Interpretation
Acinetobacter schindleri	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Staphylococcus sciuri	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Staphylococcus sarcina	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Pseudomonas aeruginosa	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Bacillus pumilus	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Enterococcus fecalis	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance

Values represent mean (± Standard deviation) measurement (mm) of the zones of inhibition on tested bacteria in response to the different concentrations of tetracycline applied. Susceptibility (≤4 µg/ml) and resistance (≥16 µg/ml) to tetracycline (CLSI M100, 28th Ed, 2018).

Table 3: Phenotypic resistance profile of 5 bacterial isolates associated with gastro-intestinal tract infection of ruminants to different concentrations of sulfonamide

	Levels of Sulphonamide application (µg/mL)					
Bacteria	0	5	10	25	50	Interpretation
Bacillus megaterium	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Arthrobacter spp.	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Pseudomonas aeruginosa	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Escherichia coli	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance
Enterococcus fecalis	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	Resistance

Values represent mean (± Standard Deviation) measurement (mm) of the zones of inhibition on tested bacteria in response to the different concentrations of sulfonamide applied. Susceptibility (≤256 µg/ml) and resistance (≥512 µg/ml) to sulphonamide (CLSI M100, 28th Ed, 2018).

Detection of tetracycline resistance (*tet*M) gene in bacteria associated with respiratory infections

Results in Figure 1 show the amplification of a tetracycline resistance gene in one of the six isolates *(E. faecalis*) with the use of the primer for *tet*M gene. The *tet*M gene in *E. faecalis* had a molecular weight of 171 bp (Lanes 18, 20 and 21).

Further effort was extended toward the evaluation of other genes like *tet*B and *tet*X that code for tetracycline resistance in other respiratory infection-associated bacterial isolates with the use of other sets of primers for their amplification. However, amplicon products for tetracycline resistance *tet*B and *tet*X genes were not detected in the bacterial isolates (Figures 2 and 3).

Detection of sulfonamide resistance (sul2) gene in bacteria associated with gastro-intestinal infections

The resistance of diarrhea-associated bacterial isolates to sulfonamide observed *in-vitro* was also confirmed after the amplification of genes that recognize sulfonamide resistance. Figure 4 shows the amplification of a sulfonamide resistance (*sul*2) gene in *Pseudomonas aeruginosa* out of the 5 isolates tested. The sulfonamide resistance gene detected in *P. aeruginosa* exhibited an amplicon size of 721 bp (Lanes 4, 6 and 15).

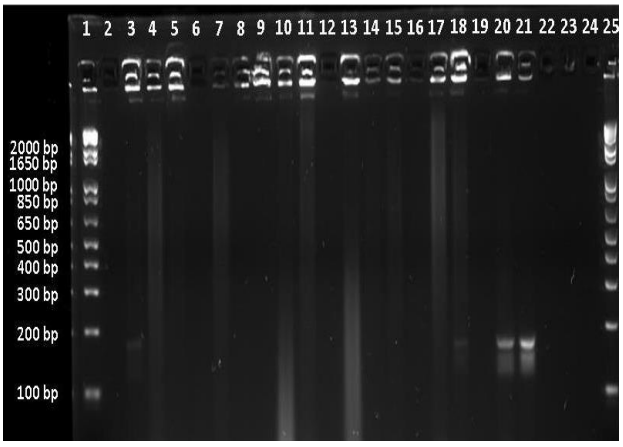

Fig. 1: Amplification of the tetracycline resistance *tet*M gene in respiratory infection-associated *E. fecalis* with the use of the specified primers. Lanes 1 and 25 (1kb+ ladder), Lanes 2 and 14 (DNA amplicons from *Arthrobacter* sp.), Lanes 3, 18, 20 and 21 (DNA amplicons from *E. faecalis*), Lanes 4 and 7 (DNA amplicons from *A. schindleri*), Lanes 5 and 9 (DNA amplicons from *P. aeruginosa),* Lanes 6, 8, 16, 17, 22, 23 and 24 (DNA amplicons from *E. coli*), Lanes 10 and 13 (DNA amplicons from *S. sciuri*), Lanes 11 and 15 (DNA amplicons from *B. pumilus*) and Lanes 12 and 19 (DNA amplicons from *S. sporosarcina).*

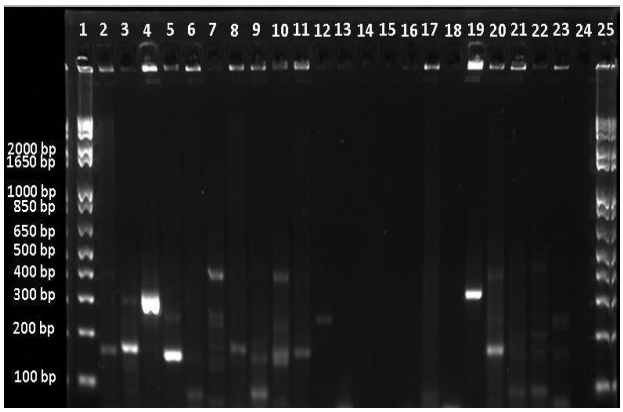

Fig. 2: Non-amplification of the tetracycline resistance *tet*B gene in respiratory infection-associated bacteria with the use of the specified primers. Lanes 1 and 25 (1kb+ ladder), Lanes 2 and 14 (DNA amplicons from *Arthrobacter* sp.), Lanes 3, 18, 20 and 21 (DNA amplicons from *E. faecalis*), Lanes 4 and 7 (DNA amplicons from *A. schindleri*), Lanes 5 and 9 (DNA amplicons from *P. aeruginosa),*Lanes 6, 8, 16, 17, 22, 23 and 24 (DNA amplicons from *E. coli*), Lanes 10 and 13 (DNA amplicons from *S. sciuri*), Lanes 11 and 15 (DNA amplicons from *B. pumilus*) and Lanes 12 and 19 (DNA amplicons from *S. sporosarcina).* The expected molecular size of the *tet*B gene is 206 bp.

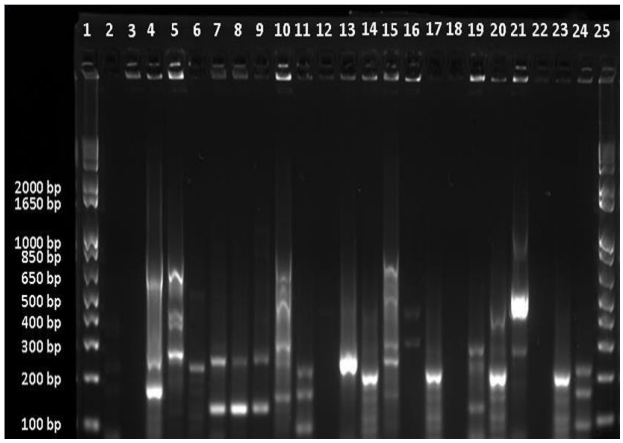

Fig. 3: Non-amplification of the tetracycline resistance *tet*X gene in respiratory infection-associated bacteria with the use of the specified primers. Lanes 1 and 25 (1kb+ ladder), Lanes 2 and 14 (DNA amplicons from *Arthrobacter* sp.), Lanes 3, 18, 20 and 21 (DNA amplicons from *E. faecalis*), Lanes 4 and 7 (DNA amplicons from *A. schindleri*), Lanes 5 and 9 (DNA amplicons from *P. aeruginosa),* Lanes 6, 8, 16, 17, 22, 23 and 24 (DNA amplicons from *E. coli*), Lanes 10 and 13 (DNA amplicons from *S. sciuri*), Lanes 11 and 15 (DNA amplicons from *B. pumilus*) and Lanes 12 and 19 (DNA amplicons from *S. sporosarcina).* The expected molecular size of the *tet*X gene is 1167 bp.

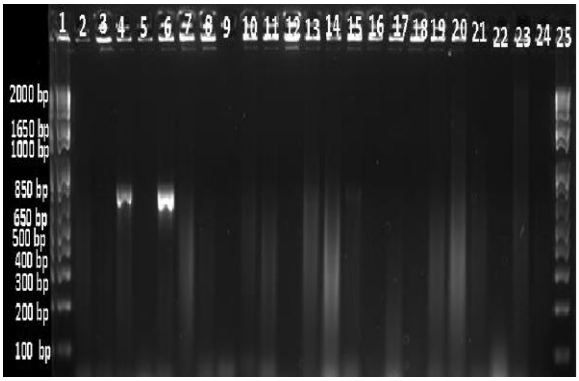

Fig. 4: Amplification of the sulfonamide resistance *sul*2 gene in gastro-intestinal infection-associated *P. aeruginosa* with the use of the specified primers. Lanes 1 and 25 (1kb+ ladder), Lanes 2, 3 and 9 (DNA amplicons from *Arthrobacter* sp.), Lanes 14, 22 and 5 (DNA amplicons from *B. megaterium*), Lanes 4, 6 and 15 (DNA amplicons from *P. aeruginosa*), Lanes 8, 19 and 23 (DNA amplicons from *E. coli) and* Lanes 7,11 and 13 (DNA amplicons from *E. faecalis).*

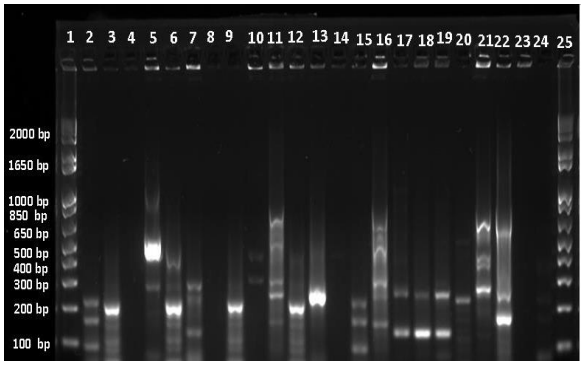

Fig. 5: Non-amplification of the sulfonamide resistance *sul*1 gene in gastro-intestinal infection-associated bacteria with the use of the specified primers. Lanes 1 and 25 (1kb+ ladder), Lanes 3, 6, 9*(*DNA amplicons from *Arthrobacter* sp.), Lanes 15, 9 and 7 (DNA amplicons from *B. megaterium*), Lanes 17, 18 and 19 (DNA amplicons from *P. aeruginosa*), Lanes 11, 16 and 21 (DNA amplicons from *E. coli),* Lanes 4, 8 and 14 (DNA amplicons from *E. faecalis).*The expected molecular size of the *sul*1 is 779 bp.

Table 4: Nucleotide sequence of the tetracycline resistance (*tet*M) gene in *E. faecalis*

Aligned sequence	Product length (bp)
AGGTTACAGAAAGCTTATTATATAACAGTGGAGCGATTACAGAATTAGGAAGCGTGGACAAAG GTACAACGAGGACGGATAATACGCTTTTAGAACGTCAGAGAGGAATTACAATCAGACAGGAAT AACCTCTTTTCAGTGGGAAAATACGAAGGTGAACATCATAGACACGCCAA	171

Table 5: Nucleotide sequence of sulfonamide resistance (*sul*2) gene in *P. aeruginosa*

Aligned sequence	Product length (bp
TCGGCATCGTCAACATAACCTCGGACAGTTTCTCCGATGGAGGCCGGTATCTGGCGCCAGACGCAG CCATTGCGCAGGCGCGTAAGCTGATGGCCGAGGGGGCAGATGTGATCGACCTCGGTCCGGCATCC AGCAATCCCGACGCCGCGCCTGTTTCGTCCGACACAGAAATCGCGCGTATCGCGCCGGTGCTGGAC GCGCTCAAGGCAGATGGCATTCCCGTCTCGCTCGACAGTTATCAACCCGCGACGCAAGCCTATGCC TTGTCGCGTGGTGTGGCCTATCTCAATGATATTCGCGGTTTTCCAGACGCTGCGTTCTATCCGCAAT TGGCGAAATCATCTGCCAAACTCGTCGTTATGCATTCGGTGCAAGACGGGCAGGCAGATCGGCGCG AGGCACCCGCTGGCGACATCATGGATCACATTGCGGCGTTCTTTGACGCGCGCATCGCGGCGCTGA CGGGTGCCGGTATCAAACGCAACCGCCTTGTCCTTGATCCCGGCATGGGGTTTTTTCTGGGGGCTG CTCCCGAAACCTCGCTCTCGGTGCTGGCGCGGTTCGATGAATTGCGGCTGCGCTTCGATTTGCCGGT GCTTCTGTCTGTTTCGCGCAAATCCTTTCTGCGCGCGCTCACAGGCCGTGGTCCGGGGGATGTCGGG GCCGCGACACTCGCTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACAAA	724

Other experiment involved evaluation of the gastrointestinal-associated bacterial isolates for sulfonamide resistance that applied a different set of primer for the amplification of another gene *sul*1. Amplicon products for sulfonamide resistance *sul*1 genes in bacterial isolates tested were not detected in any of the 5 bacterial isolates (Figure 5).

Tetracycline resistance gene in *E. fecalis* and sulfonamide resistance gene in *P. aeruginosa*

The presence of tetracycline and sulfonamide resistance in bacterial isolates was confirmed after alignment of the sequences of the tetracycline (Table 4) and sulfonamide resistance genes (Table 5). Data demonstrate that the tetracycline resistance gene detected in *E. faecalis* is made up of 171 nucleotides while that of the sulfonamide resistance gene noted in *P. aeruginosa* is comprised of 724 bp nucleotides.

DISCUSSION

Findings of the study define the presence of tetracycline resistance genes in *E. fecalis* obtained from ruminants that received treatments due to respiratory infection. Data demonstrate that resistance of *E. fecalis* to tetracycline is apparently linked to the detection of the *tet*M gene and not directly with either *tet*B or *tet*X. The involvement of *tet*M in *E. fecalis* resistance to tetracycline observed in this study implies that the functions of ribosomal protective proteins normally regulated by the gene are altered to mediate the induction of tetracycline resistance. The non-amplification of *tet*B and *tet*X genes in *E. fecalis* simply mean that neither *tet*B-mediated efflux pumps nor *tet*X-related enzymatic inactivation are involved in tetracycline resistance of the bacterium.

Results of the study demonstrated the detection of resistance genes to sulfonamide in *P. aeruginosa* isolated from ruminants that received treatments due to GI tract infections. Resistance of the bacterium to sulfonamide has been associated with the recognition of the *sul*2 gene which is also known as the dihydropteroate synthase (DHPS) gene and not with the other gene (*sul*1). The amplification of the *sul*2 gene in *P. aeruginosa* indicates the existence of mutations in the chromosome of the gene where mechanisms that mediate normal antimicrobial functions of sulfonamide are altered and neutralized bringing about

resistance to the drug. The absence of a *sul*1 gene amplicon product explain that this gene is not directly involved in sulfonamide resistance of the bacterium.

The efficiency of tetracyclines as anti-microbials has long been known in the inhibition of bacterial protein synthesis. The inhibition of bacterial protein synthesis is reportedly associated with the fusion of the antibiotic with aminoacyl-tRNA of the bacterial ribosome (Schnappinger and Hillen, 1996). The process reportedly describes the passage of tetracycline across one or more bacterial membrane systems to avert DNA transcription to m-RNA to thwart genetic information for the translation and synthesis of bacterial proteins. The trajectory of tetracycline across membrane is claimed to be energy dependent, driven by the change in pH component of the proton motive force (Nikaido and Thanassi, 1993; Schnappinger and Hillen, 1996). Inside the bacterial cytoplasm, the active tetracycline molecules are described to bind reversibly with the ribosomes forming a magnesium-tetracycline complex (Chopra *et al*., 1992; Levy, 1984). The reversible binding of tetracycline with the ribosomes reportedly clarifies the bacteriostatic effects of these antibiotics (Levy, 1984).

The mechanism that regulates bacterial resistance to tetracyclines is not easy to understand. Reports describe the involvement of efflux proteins (Tauch *et al.,* 2000), ribosomal protective proteins (Taylor and Chau, 1996; Clermont *et al.,* 1997) and enzyme inactivation (Speer *et al.,* 1991) as processes that mediate tetracycline resistance. An extensive review on the involvement of ribosomal protective protein, efflux pumps, and inactivation of enzymes in mediating tetracycline resistance was given by Chopra and Roberts (2001). Efflux genes have been associated with antibiotic resistance genes, heavy metal resistance genes and toxin production in Gram-negative bacteria (Jones *et al.,* 1992). A comparative analysis on the involvement of ribosomal protective proteins and efflux genes in mediating resistance to tetracycline has been undertaken (Sanchez-Pescador *et al.,* 1988). Inactivation of enzymes in transposons of anaerobic *Bacteroides* to effect tetracycline resistance has been described (Speer *et al*., 1991).

Of the three mechanisms that arbitrate tetracycline resistance, the one mediated by ribosomal protective protein is extensively studied. The *tet*M gene has been known as a protective protein that works in synergy with other protective proteins like *tet*O in inhibiting the destructive action of tetracycline (Chopra and Roberts, 2001) to the ribosomes, tetracycline resistance in both Gram-positive and Gram- negative species (Connell *et al.,* 2003) and in preventing the biosynthesis and release of antibiotic from the ribosomes in a reaction dependent on GTP hydrolysis (Dantley *et al*., 1998).

Sulfonamides, on the other hand, act as antimicrobials by inhibiting the synthesis of para-aminobenzoic acid (PABA) with pteridine to mediate folic acid production in bacteria. Resistance to sulphonamides is too complex to comprehend although it reportedly develops when bacterial mutations result in PABA overproduction, folic acid synthesizing enzyme protein has low affinity for sulfonamides and from a loss of cell permeability to sulfonamides (Chambers and Jawetz, 1998). Bacterial resistance to sulfonamide reportedly occurs in two pathways of mechanisms. One, is through mutation in the chromosomal dihydropteroate synthase (DHPS) gene (folp) or acquisition of an alternative DHPS gene (*sul*) whose product has a low affinity to sulphonamide (Perreten and Boerlin, 2003). Three subtypes of sul genes (*sul*1, *sul*2 and *sul*3) encode for different forms of dihydropteroate synthase gene not inhibited by sulfonamide drugs have been reported (Antunes *et al*., 2005) while three genes (*sul*1, *sul*2 and *sul*3) that encode for sulfonamide resistance have been identified (Skold, 2001; Perreten and Boerlin, 2003). The work of Enne *et al*. (2001) described the acquisition of encoding genes like *sul*1 and *sul*2 for dihydropteroate synthase (DHPS) in mediating sulfonamide resistance in bacteria. Unidentified *sul* genes aside from *sul*1, *sul*2 and *sul*3 are reported by Kashif *et al*. (2013) who studied sulfonamide resistance recognizing other mediating molecules like class 1 and class 2 integrons. Other researchers proposed the prevalence of *sul* genes in bacteria which varied depending on the environment and species of bacteria (Hindi *et al*., 2013). Wider account on the role played by *sul* genes in sulfonamide resistance is described (Wu *et al.,* 2010) where *sul1* has been found on large conjugative plasmids, on class 1 integrons and in the 3' conserve region of class 1 integron. *sul*2 was previously cited to be found on small non-conjugative plasmids and in a wide range of large conjugative plasmids. Other researchers (Byarugaba, 2004) described the association of *sul*1 genes with other resistant genes in class 1 integron while *sul*2 is located on small non-conjugative plasmids or large transmissible multi-resistant plasmid. Other reports linked *sul3* to non-classic class 1 integrons (Perreten and Boerlin, 2003) while others reported extensive and rapid spread of sulfonamide resistance in bacteria by acquisition of *sul*1, *sul*2 or *sul*3 (Trobos *et al*., 2008).

A dramatic rise in the number of drug resistant bacteria has been identified and recounted over the last four decades (Levy, 1992; and Roberts, 1991). Resistance to tetracycline was reportedly observed in *Enterococcus* sp. (Leclerc *et al*., 1996; Niemi *et al*., 1993; and Petersen *et al*., 2002), *Pseudomonas* sp. (Angus *et al*., 1982; and Yoshimura and Nikaido, 1982), *Staphylococcus* sp. (Goldstein *et al*., 1994), *Bacillus* sp. (Chopra and Roberts, 2001) and *Acinetobacter* sp. (Maleki *et al*., 2013). Repeated use of tetracycline as an important growth promotant and the acquisition of resistance genes from other species of bacteria have been cited as instruments for the development of tetracycline resistance. The study of Wang *et al*. (2014) identified sulfonamide resistant *Pseudomonas*, *Shigella* and *Bacillus* species.

The present finding which identified the *tet*M gene as the intervening factor for tetracycline resistance in *E. faecalis* isolates of ruminants is in harmony with the observations of Aarestrup (2000) who found a mobile *tet*M gene from other *Enterococcus* isolates. Other researchers described a *tet*M gene carried by transposons in *Enterococcus* and *Bacillus* species (Flannagan *et al*., 1994; Ageroso *et al*., 2002), *Streptococcus alactylicus* (Aminov *et al*., 2001), in *Streptococci*, *Neisseria* and some Gram-negative anaerobes (Villedieu, 2003) and *Listeria* species (Bertrand *et al.,* 2005).

Result of this study showed *sul*2 as the prevalent determinant of sulfonamide resistance in *P. aeruginosa*.

Previous findings demonstrated the role of the *sul*2 gene in bacterial plasmids as carriers of antibiotic resistance in *E. coli* (Enne *et al*., 2004; Bean *et al.*, 2008; Infante *et al.*, 2005; Wu *et al*., 2010), *Bacillus, Terrabacter, Agrococcus, Acinetobacter, Dietza* and *Shigella* species (Hoa *et al.*, 2010).

The present study highlighted the existence of tetracycline and sulfonamide resistance among bacteria that were associated with respiratory and gastrointestinal infections by antibiotic sensitivity testing (AST). AST is considered as an important component of veterinary diagnostics and the result of which is necessary in making decisions for treatment (Zelazny *et al*., 2005). The application of a DNA-based assay in adjunct to AST is quite indispensable in confirming antimicrobial resistance as this provides a strong basis for the selection of an alternative option for treatment or withdrawal of antibiotic use. The application of recent protocols that confirm the involvement of genes in mediating tetracycline and sulfonamide resistance in respiratory and GIT-associated bacteria of small and large ruminants attempted to define genes which carry antimicrobial resistance. A good reference in the selection for an alternative and effective treatment which least induces drug resistance in these species of animals or a basis for discontinuity of a particular drug in use may be known in the future.

Acknowledgements

Sincere thanks is extended to the Philippine Carabao Center (PCC) and the Department of Agriculture – Biotechnology Program for supporting this study. Appreciation is extended to PCC Biosafety and Environment Section staff and colleagues.

REFERENCES

Aarestrup FM, Y Ageroso, P Gerner-Smidt, M Madsen and LB Jensen, 2000. Comparison of antimicrobial resistance phenotypes and resistance genes in Enterococcus faecalis and Enterococcus faecium from humans in the community, broilers and pigs in Denmark. Diagn Microbiol Infect Dis, 37: 127-137.

Ageroso YL, B Jensen, M Givskov and M Roberts, 2002. The identification of a tetracycline resistance gene tet(M), on a Tn916-like transposon, in the Bacillus cereus group. FEMS Microbiol Letters, 214: 251-256.

Alekshun MN and SB Levy, 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell, 128: 1037-50.

Aminov RI, JC Chee-Sanford, N Garrigues, B Teferedegne, IJ Krapac, BA White and RI Mackie, 2002. Development, validation and application of PCR primers for detection of tetracycline efflux genes of Gram-negative bacteria. Appl Environ Microbiol, 68: 1786-1793.

Aminov RI, N Garrigues-Jeanjean and RI Mackie, 2001. Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl Environ Microbiol, 67: 22-32.

Angus BL, AM Carey, DA Caron, AMB Kropinski and REW Hancock, 1982. Outer membrane permeability in Pseudomonas aeruginosa: comparison of a wild-type with an antibiotic supersusceptible mutant. Antimicrob Agents Chemother, 21: 299-309.

Antunes P, J Machado and L Peixe, 2006. Characterisation of antimicrobial resistance and class 1 and 2 integrons in Salmonella enterica from different sources in Portugal. J Antimicrob Chemother, 58(2): 297-304.

Bean DC, DM Livermore and LMC Hall, 2008. Plasmid imparting sulphonamide resistance in Escherichia coli: Implications for persistence. Antimicrob Agents Chemother, 53: 1088-1093.

Bertrand S, G Huys, M Yde, D D'Haene, F Tardy, M Vrints, J Swings and J Collard, 2005. Detection and characterization of tet(M) in tetracycline-resistant Listeria strains from human and food-processing origins in Belgium and France. J Med Microbiol, 54: 1151–1156.

Byarugaba DK, 2004. A view of antimicrobial resistance in developing countries and responsible risk factor. Inter J Antimicrob Agents, 24: 105-110.

Chambers HF and E Jawetz, 1998. Sulphonamides, trimethoprim, and quinolones in basic and clinical pharmacology. In: Katzung BG Ed. Appleton-Lange pp: 761-763.

Chopra I and M Roberts, 2001. Tetracycline Antibiotics: Mode of action, applications, molecular biology and epidemiology of bacterial resistance. Microbiol Mol Biol Rev, 65: 232-260.

Clermont D, O Chesneau, G De Cespedes and T Horaud, 1997. New tetracycline resistance determinants coding for ribosomal protection in Streptococci and nucleotide sequence of tet(T) isolated from Streptococcus pyogenes A498. Antimicrob Agents Chemother, 41: 112–116.

Collignon PC, JM Conly, A Andremont, SA McEwen and A Aidara-Kane, 2016. World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies to control antimicrobial resistance from food animal production. Clin Infect Dis, 63(8): 1087–1093.

Connell SR, DM Tracz, KH Nierhaus and DE Taylor, 2003. Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob Agents Chemother, 47: 3675–3681.

Dantley KA, HK Dannelly and V Burdett, 1998. Binding interaction between Tet (M) and the ribosome: requirements for binding. J Bacteriol, 180: 4089–4092.

Enne V, PM Bennett, DM Livermore and LMC Hall, 2004. Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. J Antimicrob Chemother, 53: 958-963.

Enne V, IDM Livermore, P Stephens and LMC Hall, 2001. Persistence of sulphonamide resistance E.coli in the UK despite national prescribing restriction. Lancet, 357: 1325-1328.

Flannagan SE, LA Zitzow, YA Su and DB Clewell, 1994. Nucleotide sequence of the 18-kb conjugative transposon Tn916 from Enterococcus faecalis. Plasmid, 32: 350–354.

Garcia GG, LP Belotindos and CN Mingala, 2013. Molecular characterization of respiratory bacterial pathogens in large and small ruminants. Thai J Vet Med, 43(4): 483-489.

Garcia GG, LP Belotindos and CN Mingala, 2015. Evaluation of treatment alternatives against respiratory bacterial pathogens of small and large ruminants. Adv Envi Biol, 9(8): 149-155.

Ghosh S, MJ Sadowsky, MC Roberts, JA Gralnick and TM Lapara, 2008. Sphingobacterium sp. strain PM2-P1-29 harbours a functional tet(X) gene encoding for the degradation of tetracycline. J Appl Microbiol, 10: 1365-2672.

Goldstein FW, MD Kitzis and JF Acar, 1994. N,N-Dimethylglycyl- amido derivative of minocycline and 6-demethyl-6-deoxytetracycline, two new glycylcyclines highly effective against tetracycline-resistant gram-positive cocci. Antimicrob Agents Chemother, 38: 2218–2220.

Hindi A, E Ebtehal and S Addos, 2013. Molecular study on distribution of sul-1 and sul-2 genes among Salmonella enterica causing enteric fever. Magazin Al-Kufa Univ Biol, 5: 2.

Hoa PTH, S Managaki, N Nakada, H Takada, DH Anh, P Viet, PT Hien and S Suzuki, 2010. Abundance of sulphonamide-resistant bacteria and their resistance genes in integrated aquaculture-agriculture ponds, North Vietnam. Interdiscip Stud Environ Chem, 15-22.

Infante V, M Grape, M Larsson, C Kristiansson, L Pallecchi, GM Rossolini and G Kronvall, 2004. Acquired sulphonamide resistance genes in faecal Escherichia coli from healthy children in Bolivia and Peru. Internatl J Antimicrob Agents, 25: 308-312.

Jones CS, DJ Osborne and J Stanley, 1992. Enterobacterial tetracycline resistance in relation to plasmid incompatibility. Mol Cell Probes, 6: 313–317.

Kashifs J, R Buriros, J Memon, M Yaqoob, J Soomros, D Dongxue, H Jinhu and W Liping, 2013. Detection of class 1 and 2 integrons, β-lactamase genes and molecular characterization of sulphonamide resistance in Escherichia coli isolates recovered from poultry in China. Pakistan Vet J, 33(3): 321-324.

Kordick DL, MG Papich and EB Breitschwerdt, 1997. Efficacy of enrofloxacin or doxycycline for treatment of Bartonella henselae or Bartonella clarridgeiae infection in cats. Antimicrob Agents Chemother, 41: 2448–2455.

Lanz R, P Kuhnert and P Boerlin, 2003. Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. Vet Microbiol, 91: 73-84.

Leclerc H, LA Devriese and DAA Mossel, 1996. Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. J Appl Bacteriol, 81: 459-466.

Levy SB, 1984. Resistance to the tetracyclines. In: LE Bryan Ed. Antimicrobial Drug Resistance. Academic Press, Orlando, Florida, USA. pp. 191–240.

Levy SB, 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob Agents Chemother, 36: 695–703.

Maleki MH, Z Sekawi, S Soroush, F Azizi-jalilian, KH Asadollahi, S Mahammadi, M Emaneini and M Taherikalani, 2013. Phenotypic and genotypic characteristics of tetracycline resistant in Acinetobacter baumanni isolates from nosocomial infections at Tehran hospitals. Iran J Basic Med Sci, 17: 21-26.

Messi P, E Guerrieri and M Bondi, 2005. Antibiotic resistance and antibacterial activity in heterotrophic bacteria of mineral water origin. Sci Total Environ, 346: 213-219.

Niemi RM, SI Niemela, DH Bamfort, J Hantula, T Hyvarinen, T Forsten and A Raateland, 1993. Presumptive fecal streptococci in environmental samples characterized by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Appl Environ Microbiol, 59: 2190-2196.

Nikaido H and DG Thanassi, 1993. Penetration of lipophilic agents with multiple protonation sites into bacterial cells: Tetracyclines and Fluoroquinolones as examples. Antimicrobial Agents Chemother, 37: 1393–1399.

Perreten V and P Boerlin, 2003. A new sulphonamide resistant gene (Sul3) in Escherichia coli is widespread in the pig population of Switzerland. Antimicrob Agents Chemother, 47: 1162-1172.

Petersen A, JS Andersen, T Kaewmak, T Somsiri and A Dalsgaard, 2002. Impact of integrated fish farming on antimicrobial resistance in a pond environment. Appl Environ Microbiol, 68: 6036-6042.

Reynolds R, J Shackcloth, D Felmingham and A McGowan, 2003. Antimicrobial susceptibility of lower respiratory tract pathogens in Great Britain and Ireland 1999-2001 related demographic and geographical: BSAC Respiratory Resistance Surveillance Programme. J Antimicrob Chemother, 52: 931-943.

Roberts MC, 1991. Characterization of the tetM determinant in urogenital and respiratory bacteria. Antimicrob Agents Chemother, 34: 476–478.

Sanchez-Pescador R, JT Brown, M Roberts and MS Urdea, 1988. Homology of the tetM with translational elongation factors: Implications for potential modes of tetM conferred tetracycline resistance. Nucleic Acids Res, 16: 1218.

Schnappinger D and W Hillen, 1996. Tetracyclines: Antibiotic action, uptake and resistance mechanisms. Arch Microbiol, 165: 359–369.

Skold O, 2001. Resistance to trimethoprim and sulphonamide. Vet Res, 32: 261-273.

Speer BS, L Bedzyk and AA Salyers, 1991. Evidence that a novel tetracycline resistance gene found on two Bacteroides transposons encodes an NADP-requiring oxido-reductase. J Bacteriol, 173: 176–183.

Tauch A, A Puhler, J Kalinowski and G Thierbach, 2000. Tet Z, a new tetracycline resistance determinant discovered in Gram-positive bacteria shows high homology to Gram-negative regulated efflux systems. Plasmid, 44: 285–291.

Taylor DE and A Chau, 1996. Tetracycline resistance mediated by ribosomal protection. Antimicrob Agents Chemother, 40: 1–5.

Trobos M, L Jakobsen, KE Olsen, NF Rimodt-Moller, AM Hammerum, K Pedersen, Y Agerso, LJ Porsbo and JE Olsen, 2008. Prevalence of sulphonamide resistance and class 1 integron genes in Escherichia coli isolates obtained from broilers, broiler meat, healthy humans

and urinary infections in Denmark. Internatl J Antimicrob Agents, 32: 367-369.

Villedieu A, ML Diaz-Torres, N Hunt, R McNab, DA Spratt, M Wilson and P Mullany, 2003. Prevalence of tetracycline resistance genes in oral bacteria. Antimicrob Agents Chemother, 3: 878–882.

Wang N, X Yang, S Jiao, J Zhang, B Ye, S Gao, 2014. Sulfonamide-resistant bacteria and their resistance genes in soils fertilized with manures from Jiangsu Province, Southeastern China. PLoS One, 9(11): e112626.

Wu S, A Dalsgaard, AM Hammerum, LJ Porsbo and L Jensen, 2010. Prevalence and characterization of plasmid carrying sulphonamide resistance genes among Escherichia coli from pigs, pig carcasses and human. Acta Vet Scand, 52: 47.

Yoshimura F and H Nikaido, 1982. Permeability of Pseudomonas aeruginosa outer membrane to hydrophilic solutes. J Bacteriol 152: 636-642.

Zelazny AM, MJ Ferraro, A Glennen, JF Hindler, LM Mann, S Munro, PR Murray, LB Reller, FC Tenover and JH Jorgensen, 2005. Selection of strains for quality assessment of the disk induction method for detection of inducible clindamycin resistance in staphylococci: a CLSI collaborative study. J Clin Microbiol, 43: 2613–2615.