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Impact of veterinary antibiotics on plasmid-encoded antibiotic resistance transfer

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Objectives: Resistance genes can be genetically transmitted and exchanged between commensal and pathogenic bacterial species, and in different compartments including the environment, or human and animal guts (One Health concept). The aim of our study was to evaluate whether subdosages of antibiotics administered in veterinary medicine could enhance plasmid transfer and, consequently, resistance gene exchange in gut microbiota.

Methods: Conjugation frequencies were determined with *Escherichia coli* strains carrying IncL- (bla_{OXA-48}) or IncI1-type ($bla_{CTX-M-1}$) plasmids subjected to a series of subinhibitory concentrations of antibiotics used in veterinary medicine, namely amoxicillin, ceftiofur, apramycin, neomycin, enrofloxacin, colistin, erythromycin, florfenicol, lincomycin, oxytetracycline, sulfamethazine, tiamulin and the ionophore narasin. Treatments with subinhibitory dosages were performed with and without supplementation with the antioxidant edaravone, known as a mitigator of the inducibility effect of several antibiotics on plasmid conjugation frequency (PCF). Expression of SOS-response associated genes and fluorescence-based reactive oxygen species (ROS) detection assays were performed to evaluate the stress oxidative response.

Results: Increased PCFs were observed for both strains when treating with florfenicol and oxytetracycline. Increased expression of the SOS-associated *recA* gene also occurred concomitantly, as well as increased ROS production. Addition of edaravone to the treatments reduced their PCF and also showed a decreasing effect on SOS and ROS responses for both plasmid scaffolds.

Conclusions: We showed here that some antibiotics used in veterinary medicine may induce transfer of plasmid-encoded resistance and therefore may contribute to the worldwide spread of antibiotic resistance genes.

Introduction

Antimicrobials are important global resources and have been widely used, from treating human and animal infections to ensuring livestock production and maintaining health and productivity for the farmers.[1] Genes encoding antibiotic resistance can be genetically transmitted and therefore exchanged between different bacterial species cohabiting the environment, human and farm animal communities, and companion animals.[2]

Antimicrobial resistance is one of the most serious global public health concerns according to the WHO. The WHO coordinated an action plan including strategies to optimize the use of antibiotics in humans as well as in animals.[3] To support this plan, the World Organisation for Animal Health (WOAH, formerly OIE) also developed a strategy for the prudent use and monitoring the volume of antimicrobial usage.[4] Together, WHO and WOAH aim to control the massive usage of antibiotics, preventing dissemination of resistance genes.[3,4]

Resistance to antibiotics is a great concern when considering *Escherichia coli*, a commensal microorganism that inhabits the human and animal gut microbiota, but can also be an opportunistic pathogen harbouring essential virulence genes. It may be a source of resistance genes for other pathogenic *E. coli* or different bacterial species.[5,6] As a human pathogen, it is responsible for causing urinary and gastrointestinal tract infections, bloodstream infections, meningitis and septicaemia.[7] In addition, due to its versatile nature it can be a significant pathogen for animals, being the main source of

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diarrhoea.[8] *E. coli* is therefore the 'One Health' pathogen par excellence. Consequently, it is important to evaluate whether antibiotic selective pressure in the veterinary world might significantly impact the *E. coli* ecology and resistance phenotype in animals and, in a more complex way, in humans, as a consequence of direct or indirect transmissions of resistance strains but also of resistance plasmids.

ESBLs of the CTX-M type are the most commonly identified ESBLs among animal isolates, with the $bla_{CTX\text{-}M\text{-}1}$ gene often being detected on IncI plasmids.[9] The latter are mainly identified in *E. coli* and *Salmonella* spp., being frequently isolated from humans and poultry, but also from pigs, cattle, dogs and other animals.[10] These plasmids may occasionally carry the ESBL gene $bla_{TEM\text{-}52}$ and are frequently associated with *E. coli* ST10 in livestock.[11] Belonging to the same incompatibility family, the IncI-γ plasmids are associated with other resistance determinants, such as the a commonly identified $bla_{CMY\text{-}2}$ β-lactamase gene.[12,13] Nevertheless, evaluating the exchange of ESBLs within humans, animals and the environment requires an integrative concept, which still generates conflicting results, being subjected to a series of difficulties such as the selection and evaluation of a trustful representative population of each ecosystem, whether a strain can colonize different ecosystems, and phylogenetics/evolution of core and accessory genomes in each community.[14]

The acquisition of acquired resistance mechanisms through horizontal gene transfer (HGT), mainly due to plasmid acquisition, has been shown to occur in different stress conditions, such as alterations of ion concentrations, extreme temperature changes, starvation conditions or exposure to antibiotics (non-optimal dosage for the selected microorganism).[15,16] These stress conditions may, therefore, in some instances, be considered as regulatory elements, either leading to increased or decreased resistance transfer rates.[15,16]

Stress induces reactive oxygen species (ROS) production, contributing to the activation of the SOS-mediated response in bacteria. In *E. coli*, oxidative metabolism can cause deleterious consequences through DNA mutations.[17] The SOS system is a damage-response pathway triggered by the RecA protein (encoded by the *recA* gene), role of which is crucial in the survival and repair of bacterial DNA.[18] Although the SOS regulon is composed of more than 40 genes with different functions (e.g. translation synthesis, repair, recombination) in *E. coli*,[18] RecA plays an important role, being a multifunctional protein acting not only on promoting homologous recombination and DNA repair, but also in the induction of biofilm formation and HGT.[19] Thus, stress conditions may act as inducers, enhancing mutagenesis and HGT by activating the ROS/SOS response, eventually contributing to the spread of resistance genes.

The goal of this study was to evaluate whether subdosage of antibiotics used in veterinary medicine could enhance plasmid transfer between *E. coli* isolates and, consequently, resistance gene exchange in animal gut microbiota. In addition, since edaravone was previously shown to reduce the plasmid conjugation frequency (PCF) in an IncF-like incompatibility family plasmid (pOX38) in human *E. coli* isolates,[20] this molecule was also tested here as a potential mitigator of the plasmid dissemination.

Materials and methods

Bacterial strains and plasmids

Two *E. coli* strains were used as plasmid donors in our conjugation experiments, namely (i) a clinical isolate harbouring an IncL plasmid (pOXA48a) carrying the $bla_{OXA\text{-}48}$ carbapenemase gene;[21] and (ii) an isolate recovered from poultry, harbouring an IncI1 plasmid carrying the ESBL gene $bla_{CTX\text{-}M\text{-}1}$.[22] The nalidixic acid-resistant *E. coli* strain JM109 was used as the recipient in conjugation experiments.[23]

Antibiotics and antimicrobial susceptibility testing

Antibiotics representing the most often administered classes in veterinary medicine were selected for this study, including the β-lactams amoxicillin and ceftiofur (an extended-spectrum cephalosporin specifically used in animals), the aminoglycosides apramycin and neomycin, the fluoroquinolone enrofloxacin, the polymyxin colistin, the macrolide erythromycin, the phenicol florfenicol, the lincosamide lincomycin, the cycline oxytetracycline, the sulphonamide sulfamethazine, the pleuromutilin tiamulin and the ionophore narasin. All were sourced from Sigma–Aldrich (St. Louis, USA) except erythromycin (Acros Organics, Waltham, USA) and neomycin (Apollo Scientific, Bredbury, UK). The microdilution method was performed in Mueller–Hinton medium (MH) (Bio-Rad, Cressier, Switzerland) to determine the MIC according to the Veterinary Committee on Antimicrobial Susceptibility Testing (VetCAST) and CLSI for bacteria isolated from animals (CLSI VET) recommendations and guidelines.[24–26]

Conjugation assays

Experiments were performed as described in our recent former study,[20] with adjustments, summarized as: (i) plasmid donors and recipient were pre-inoculated in LB (Bio-Rad, Cressier, Switzerland) overnight at 37°C with shaking; (ii) they were then incubated in LB at 37°C with shaking for an additional 5 h period. At this time, donors were in the presence or absence of subinhibitory antibiotic concentrations (half of the MIC corresponding to each donor) and conjugation inhibitor edaravone 0.1 mM (3-methyl-1-phenyl-2-pyrazolin-5-one; Sigma–Aldrich, St. Louis, USA); (iii) cells were centrifuged at 3000×*g* for 10 min and resuspended in the residual medium (3 mL) after pouring off the supernatant; (iv) donors and recipients were respectively mixed in a 1:4 volume ratio and centrifuged at 3000×*g* for 10 min before being transferred onto filters (0.22 mm; Merck Millipore, Ireland) and incubated in LB plates (Carl Roth, Karlsruhe, Germany) at 37°C for 4 h; (v) cells were washed from the filters using NaCl and the mixture was vortexed to stop the conjugation step; and (vi) serial dilution was performed and the mixture was plated onto LB agar containing 50 mg/L ampicillin (isolate carrying the $bla_{CTX\text{-}M\text{-}1}$ gene) or temocillin (isolate carrying the $bla_{OXA\text{-}48}$ gene) for quantifying donors and transconjugants and 50 mg/L ampicillin or temocillin plus 50 mg/L nalidixic acid (for quantifying transconjugants only) and finally incubated overnight at 37°C.

Conjugation frequencies (CFs) were calculated by dividing the number of transconjugants by the number of donors.

Fluorescent ROS detection

Donors were submitted to treatment with and without compounds and incubated at 37°C for 5 h with shaking. After that, the ROS detection assay was done as described by Castro-Alférez *et al.*,[27] with adjustments. Measurements of chemical hydrolysis of the probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma–Aldrich, St. Louis, USA) in the fluorescent compound 2,7-dichlorofluorescein (DCF) were detected by fluorescence spectroscopy at 522 and 498 nm emission and excitation wavelengths, respectively. Fluorescence detection was performed in 96-well solid-bottom black plates using the

TECAN 200 Pro (Tecan, Männedorf, Switzerland) fluorimeter. H_2O_2 at 2.5 mM was used as a positive control for the experiment.

mRNA extraction and cDNA synthesis

After being submitted to the initial treatment with or without compounds and incubated at 37°C for 5 h with shaking, total RNA was extracted using the Quick-RNA™ Miniprep kit (Zymo Research, Irvine, USA). The Turbo DNA-*free*™ kit (Invitrogen, Waltham, USA) was then used to remove contaminating DNA from RNA preparations, and DNase and divalent cations from the samples. cDNA synthesis was done using the LunaScript® RT SuperMix kit (New England Biolabs, Ipswich, USA). All experiments were performed following the manufacturers' instructions. Finally, cDNA samples concentrations were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

Quantitative RT–PCR (RT-qPCR)

RT–qPCR experiments were performed using the Rotor-Gene Q cycler (QIAGEN, Hilden, Germany). Primers targeting the 16S rRNA-encoding gene (reference), the recombinase *recA* gene, and the cell division inhibitory gene *sfiA* were used in the experiments.[28] Reactions were set up in a total volume of 20 µL with a GoTaq® qPCR Master Mix kit (Promega, Madison, USA). The cycle threshold (C_T) values were analysed by the $2^{-\Delta\Delta CT}$ method.[29] Relative expression levels were calculated by comparison with the control samples and the condition values were corrected with the appropriate reference gene.

Statistical analyses

All experiments were performed in three independent replicates. Data were analysed by one-way analysis of variance (ANOVA) corrected for multiple comparisons using statistical hypothesis testing with Tukey using GraphPad Prism Software version 9.3.1. Statistically significant results were defined with a confidence level of 95% ($P < 0.05$).

Results

Increased PCFs upon supplementation with subinhibitory concentrations of florfenicol and oxytetracycline

Antibiotics used in veterinary medicine were tested to evaluate their capability of modifying PCFs. *E. coli* JM109 was used as recipient for the IncL plasmid bearing the bla_{OXA-48} gene and IncI1 harbouring the $bla_{CTX-M-1}$ gene; both *E. coli* donors were treated in the presence of ½ of the MIC concentration for the different antibiotics with or without the antioxidant edaravone.

Conjugation experiments revealed an increased PCF for both IncL and IncI1 plasmids when treated with florfenicol and oxytetracycline. The fold changes for conjugation of *E. coli* containing IncL plasmids were 13.7- and 97.2-fold, respectively, for florfenicol and oxytetracycline, while being 17.2- and 8.9-fold for the IncI1 plasmid. These results were achieved after comparison with the control experiment performed in the absence of antibiotics for each strain and can be seen in Table 1 and Table 2.

By contrast, no significant increase of PCF was observed after supplementation with subinhibitory concentrations of amoxicillin, apramycin, ceftiofur, colistin, enrofloxacin, lincomycin, narasin, sulfamethazine, tiamulin, erythromycin and neomycin, in comparison with controls.

Mitigation of the inducible effect on PCF upon supplementation with the antioxidant edaravone

Since increased PCFs were observed for both the IncL and IncI1 plasmids in the presence of florfenicol and oxytetracycline, the effect of edaravone as a potential reducer of plasmid transfer was tested. Indeed, edaravone supplementation was shown to counteract the ROS production, a phenomenon speculated to be associated with the exchange of bacterial plasmids while in stress conditions.

Table 1. Conjugation frequency and fold change in filter-mating assays for OXA-48-producing *E. coli* carrying an IncL plasmid

		Control		½ MIC		½ MIC + EDA		
Compounds	MIC (mg/L)	CF	FC	CF	FC	CF	FC	FC*
Amoxicillin	≥1024	$1.00\times10^{0}\pm4.98\times10^{-1}$	1	$7.60\times10^{-1}\pm7.38\times10^{-1}$	0.8	$1.06\times10^{0}\pm1.45\times10^{0}$	1.1	−0.3
Ceftiofur	4	$1.17\times10^{0}\pm7.47\times10^{-1}$	1	$4.10\times10^{0}\pm1.74\times10^{0}$	4.1	$2.65\times10^{0}\pm2.03\times10^{0}$	2.7	1.5
Neomycin	2	$1.00\times10^{0}\pm4.98\times10^{-1}$	1	$1.49\times10^{0}\pm1.32\times10^{0}$	1.5	$1.60\times10^{0}\pm2.27\times10^{0}$	1.6	−0.1
Apramycin	4	$1.00\times10^{0}\pm4.98\times10^{-1}$	1	$3.12\times10^{0}\pm4.93\times10^{0}$	3.1	$1.01\times10^{0}\pm1.16\times10^{0}$	1.0	3.1
Oxytetracycline	1	$9.99\times10^{-1}\pm7.77\times10^{-1}$	1	$9.73\times10^{1}\pm9.24\times10^{0}$	**97.2**	$4.09\times10^{1}\pm8.62\times10^{0}$	**40.9**	**2.4↓**
Enrofloxacin	0.016	$9.63\times10^{-1}\pm9.34\times10^{-1}$	1	$1.26\times10^{0}\pm1.13\times10^{0}$	1.3	$6.85\times10^{-1}\pm4.60\times10^{-1}$	0.7	0.8
Florfenicol	8	$6.68\times10^{-1}\pm1.78\times10^{-1}$	1	$1.37\times10^{1}\pm6.96\times10^{0}$	**13.7**	$1.76\times10^{0}\pm1.36\times10^{-1}$	**1.8**	**7.6↓**
Lincomycin	≥1024	$9.99\times10^{-1}\pm7.77\times10^{-1}$	1	$6.25\times10^{0}\pm6.84\times10^{0}$	6.2	$3.10\times10^{0}\pm3.64\times10^{0}$	3.1	2.0
Sulfamethazine	1024	$9.99\times10^{-1}\pm7.77\times10^{-1}$	1	$4.32\times10^{0}\pm3.27\times10^{0}$	4.3	$3.86\times10^{0}\pm3.17\times10^{0}$	3.8	1.1
Colistin	0.0312	$1.00\times10^{0}\pm8.19\times10^{-1}$	1	$1.79\times10^{0}\pm6.13\times10^{-1}$	1.8	$1.74\times10^{0}\pm1.83\times10^{0}$	1.7	0.1
Narasin	128	$9.99\times10^{-1}\pm7.77\times10^{-1}$	1	$4.91\times10^{0}\pm2.89\times10^{0}$	4.9	$3.13\times10^{0}\pm8.05\times10^{-1}$	3.1	1.6
Tiamulin	256	$6.68\times10^{-1}\pm1.78\times10^{-1}$	1	$7.68\times10^{0}\pm4.44\times10^{0}$	7.7	$2.79\times10^{0}\pm3.65\times10^{0}$	2.8	2.7
Erythromycin	64	$1.00\times10^{0}\pm4.98\times10^{-1}$	1	$1.86\times10^{0}\pm3.08\times10^{0}$	1.9	$2.98\times10^{-1}\pm3.79\times10^{-1}$	0.3	6.3

CF, conjugation frequency; FC, fold change; FC*, fold change between treated with antibiotic + EDA versus treated with antibiotic only; EDA, edaravone (0.1 mM). Bold indicates statistically significant results ($P < 0.05$). Downward arrows indicate decrease in fold change for treated with antibiotic + EDA versus treated with antibiotic only.

A reduction in the increased PCF was observed during our conjugation experiments for both plasmids when treated with the antibiotics that were previously shown to exhibit induction effects. By contrast, no PCF induction effect was observed for strains treated with edaravone plus antibiotics when compared with those treated only with antibiotic.

For the IncL plasmid, florfenicol- and oxytetracycline-induced conjugation rates were significantly reduced to 7.6- and 2.4-fold, respectively, when edaravone was added to the treatment (Table 1). For the IncI1 plasmid, both induced conjugations were significantly reduced to 8.2- and 3.6-fold for florfenicol and oxytetracycline, respectively (Table 2).

Enhanced oxidative stress response after treatment with subinhibitory concentrations of florfenicol and oxytetracycline

ROS production assays were performed with both *E. coli* plasmids (IncI1 and IncL) grown with florfenicol and oxytetracycline subinhibitory concentrations. Supplementation with neomycin was used as a negative control for ROS assays, considering that this antibiotic did not show any impact on the PCF during previous experiments.

Results of ROS production showed a correlation with conjugation assays for the selected antibiotics. A significant increase of 3.9- ($P \leq 0.0001$) and 3.2-fold ($P \leq 0.001$) in the ROS production was observed after treatment with florfenicol and oxytetracycline, respectively, for the *E. coli* carrying an IncL plasmid when compared with the control (Figure 1a). A decrease of 1.4-fold was observed when edaravone was added to the treatment with florfenicol ($P < 0.05$), compared with the florfenicol alone (Figure 1b). Opposite to florfenicol, when edaravone was added to the treatment with oxytetracycline, no change was observed in ROS production when comparing with oxytetracycline alone ($P = 0.94$) (Figure 1c).

For the *E. coli* carrying the IncI1 plasmid, a significant increase of the ROS production was observed in the presence of florfenicol and oxytetracycline, being 7.2- ($P \leq 0.005$) and 24.8-fold ($P \leq 0.0001$), respectively, when compared with the control (Figure 1d). A decrease of 1.7-fold ($P < 0.05$) was detected when edaravone was added to florfenicol in the treatment (Figure 1e) when compared with florfenicol alone. On the contrary to that observed in the isolate carrying IncL, when edaravone was added to oxytetracycline the IncI1 isolate presented a significant reduction of 2.2-fold ($P \leq 0.0001$) in ROS production when compared with oxytetracycline treatment alone (Figure 1f). Lastly, as expected, neomycin and edaravone (alone) treatments did not present any effect on the ROS production for both *E. coli* isolates.

Antibiotic subdosage treatment triggers typical SOS gene expression

The expression levels of *recA* and *sfiA* genes were measured in both *E. coli* isolates (IncL and IncI1) after treatment with or without subinhibitory concentrations of florfenicol and oxytetracycline, with or without edaravone. Supplementation with neomycin was also considered here as a negative control for the RT–qPCR assay.

Significant increases of 2.5- and 1.6-fold of *recA* expression were observed for the *E. coli* (IncL) after treatment with florfenicol and oxytetracycline, respectively. Conversely, significant decreases were observed after adding edaravone to florfenicol and oxytetracycline. In contrast to *recA*, no differences in *sfiA* expression levels were observed with all treatments for the *E. coli* (IncL) strain (Table 3).

The expression of the *recA* gene was significantly increased in *E. coli* (IncI1) with florfenicol and oxytetracycline, 2.9- and 3-fold change being respectively observed, as compared with the control. Adding edaravone, a significant decrease of 3.9-fold was

Table 2. Conjugation frequency and fold change in filter-mating assays for CTX-M-1-producing *E. coli* carrying an IncI1 plasmid

		Control		½ MIC		½ MIC + EDA		
Compounds	MIC (mg/L)	CF	FC	CF	FC	CF	FC	FC*
Amoxicillin	≥1024	$9.99 \times 10^{-1} \pm 1.10 \times 10^{0}$	1	$2.29 \times 10^{0} \pm 1.81 \times 10^{0}$	2.3	$1.93 \times 10^{0} \pm 1.44 \times 10^{0}$	1.9	0.2
Ceftiofur	256	$9.99 \times 10^{-1} \pm 1.10 \times 10^{0}$	1	$6.75 \times 10^{-1} \pm 6.85 \times 10^{-1}$	0.7	$9.75 \times 10^{-2} \pm 7.33 \times 10^{-2}$	0.1	7
Neomycin	2	$9.99 \times 10^{-1} \pm 1.10 \times 10^{0}$	1	$1.06 \times 10^{0} \pm 9.20 \times 10^{-1}$	1.1	$5.83 \times 10^{-1} \pm 8.37 \times 10^{-1}$	0.6	0.8
Apramycin	8	$1.00 \times 10^{0} \pm 1.56 \times 10^{-0}$	1	$1.34 \times 10^{0} \pm 1.78 \times 10^{0}$	1.3	$3.53 \times 10^{-1} \pm 3.49 \times 10^{-1}$	0.4	3.2
Oxytetracycline	512	$1.00 \times 10^{0} \pm 1.46 \times 10^{-0}$	1	$8.91 \times 10^{0} \pm 4.32 \times 10^{0}$	**8.9**	$2.51 \times 10^{0} \pm 1.70 \times 10^{0}$	**2.5**	**3.6↓**
Enrofloxacin	0.0312	$9.99 \times 10^{-1} \pm 1.10 \times 10^{0}$	1	$2.46 \times 10^{0} \pm 6.21 \times 10^{-1}$	2.5	$1.03 \times 10^{0} \pm 3.61 \times 10^{-1}$	1.0	2.5
Florfenicol	8	$1.00 \times 10^{0} \pm 9.74 \times 10^{-1}$	1	$1.72 \times 10^{1} \pm 4.63 \times 10^{0}$	**17.2**	$2.12 \times 10^{0} \pm 1.21 \times 10^{0}$	**2.1**	**8.2↓**
Lincomycin	≥1024	$1.00 \times 10^{0} \pm 1.56 \times 10^{-0}$	1	$3.54 \times 10^{0} \pm 5.10 \times 10^{0}$	3.5	$5.08 \times 10^{-1} \pm 4.95 \times 10^{-1}$	0.5	7
Sulfamethazine	1024	$1.00 \times 10^{0} \pm 1.56 \times 10^{-0}$	1	$1.66 \times 10^{0} \pm 2.27 \times 10^{0}$	1.7	$6.63 \times 10^{-1} \pm 9.57 \times 10^{-1}$	0.7	2.4
Colistin	0.0312	$1.00 \times 10^{0} \pm 1.12 \times 10^{0}$	1	$1.34 \times 10^{0} \pm 7.52 \times 10^{-1}$	1.3	$1.14 \times 10^{0} \pm 8.65 \times 10^{-1}$	1.1	0.2
Narasin	128	$1.00 \times 10^{0} \pm 1.56 \times 10^{-0}$	1	$1.49 \times 10^{0} \pm 2.22 \times 10^{0}$	1.5	$2.36 \times 10^{-1} \pm 1.02 \times 10^{-1}$	0.2	7.5
Tiamulin	128	$1.00 \times 10^{0} \pm 9.74 \times 10^{-1}$	1	$9.12 \times 10^{-1} \pm 9.03 \times 10^{-1}$	0.9	$1.88 \times 10^{0} \pm 9.38 \times 10^{-1}$	1.9	−0.5
Erythromycin	64	$9.99 \times 10^{-1} \pm 1.10 \times 10^{0}$	1	$4.60 \times 10^{-1} \pm 3.47 \times 10^{-1}$	0.5	$3.17 \times 10^{-1} \pm 8.37 \times 10^{-1}$	0.4	0.2

CF, conjugation frequency; FC, fold change; FC*, fold change between treated with antibiotic + EDA against treated with antibiotic only; EDA, edaravone (0.1 mM). Bold indicates statistically significant results ($P < 0.05$). Downward arrows indicate decrease in fold change for treated with antibiotic + EDA versus treated with antibiotic only.

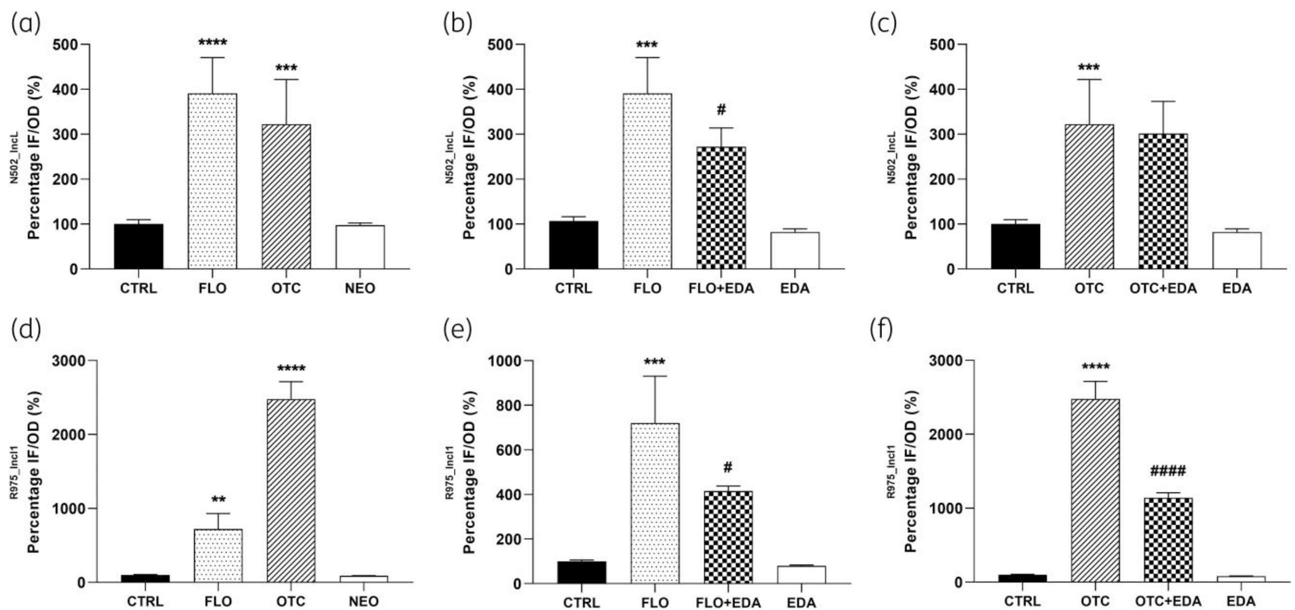

Figure 1. ROS experiment by fluorimetry. (a) ROS production for *E. coli* (IncL) after treatment with antibiotics; (b) ROS production for *E. coli* (IncL) after incubation with florfenicol, with and without edaravone; (c) ROS production for *E. coli* (IncL) after incubation with oxytetracycline, with and without edaravone; (d) ROS production for *E. coli* (IncI1) after treatment with antibiotics; (e) ROS production for *E. coli* (IncI1) after incubation with florfenicol, with and without edaravone; (f) ROS production for *E. coli* (IncI1) after incubation within oxytetracycline, with and without edaravone. IF/OD, intensity of fluorescence/OD; CTRL, control (no treatment); FLO, florfenicol (4 mg/L for both isolates); OTC, oxytetracycline (IncL, 0.5 mg/L; IncI1, 256 mg/L), NEO, neomycin (1 mg/L for both isolates); EDA, edaravone (0.1 mM). Data are presented as mean and SD. */#, $P<0.05$ (*, against the control; #, against the treated only with antibiotics); **/##, $P\leq0.01$; ***/###, $P\leq0.001$; ****/####, $P\leq0.0001$.

detected in *recA* expression when considering the florfenicol treatment. By contrast, we did not observe any decrease in the combined treatment of oxytetracycline+edaravone ($P=0.98$). The *sfiA* gene expression level was increased 11.1-fold following the oxytetracycline treatment, and no decreasing effect could be observed with the oxytetracycline+edaravone treatment ($P=0.99$). No significant effect was observed for the expression of the same gene when *E. coli* was treated with florfenicol. Lastly, as expected, neomycin and edaravone (alone) treatments did not show any effect on the expression levels of SOS-response associated genes for both *E. coli* isolates (Table 4).

Discussion

It is now well admitted that the problem of antimicrobial resistance, particularly associated with *E. coli* species, is not limited to human clinical isolates and that extra sources such as food products,[22,30,31] companion animals[32] and the environment[33] also represent a major source of concern. Our study was designed to evaluate the possible impact that antibiotics used in veterinary practice may have on the transfer of plasmids encoding the main antibiotic resistance determinants identified in animals and that might possibly be further transferred to human pathogens.

We showed here that florfenicol and oxytetracycline, inhibitors of bacterial protein synthesis, had a significant induction effect on conjugation frequencies in *E. coli*. Our experiments showed that this effect was likely associated with the SOS response and ROS production. Florfenicol is widely used for treating infections in cattle, fish, pigs and poultry, as well as a feed additive in farm animals in certain geographical areas, and resistance to this antibiotic has been extensively reported during recent decades.[34] Reports about changes in PCF triggered by the activation of SOS response proteins when treating *E. coli* with subinhibitory dosage of phenicols such as florfenicol remain rare. Bethke *et al.*[35] detected an increase in PCF when *E. coli* was exposed to chloramphenicol, another phenicol, but its use is only for human medicine, and it has been partially abandoned in Europe (but not in the USA) because of its association with fatal aplastic anaemia.[36] These data are in line with our results, even though they were observed with plasmids possessing different scaffolds. In contrast to our observations, the same study identified an increased PCF when erythromycin was used.[35] However, the authors of the aforementioned study demonstrated an increased PCF induced by erythromycin for only one of the several plasmid scaffolds tested, an IncFIB/IncFII/Col156 plasmid,[35] different from the ones used in the present study (IncI1 and IncL). Thus, the potential effect of antibiotics on PCF may depend on the plasmid incompatibility group.

In that latter study, supplementation with kanamycin did not present any effect on the PCF in *E. coli*, and the authors speculated that such a lack of effect could be associated with the mechanism of action of this antibiotic, which targets the 30S ribosomal subunit.[35] In our study, oxytetracycline, belonging to another class of antibiotics but also targeting the 30S ribosomal subunit, promoted a significant enhancement in the conjugation frequencies for both IncI1 and IncL plasmids, being more accentuated in the latter one. Oxytetracycline has been massively used to treat infections in cattle, fish, pigs, poultry and others and,

Table 3. Expression of chromosomal genes related to SOS response in OXA-48-producing *E. coli* carrying an IncL plasmid when submitted to subinhibitory concentrations of different compounds

	$2^{-\Delta\Delta CT}$						
Gene	C	FLO	FLO + EDA	OTC	OTC + EDA	NEO	EDA
recA	1	**2.46 ± 0.39**	**1.42 ± 0.16**	**1.56 ± 0.14**	**1.22 ± 0.02**	1.29 ± 0.42	0.85 ± 0.48
sfiA	1	0.31 ± 0.08	0.26 ± 0.03	0.59 ± 0.13	0.73 ± 0.13	1.40 ± 0.44	0.70 ± 0.16

FLO, florfenicol (4 mg/L); OTC, oxytetracycline (0.5 mg/L); AMX, amoxicillin (512 mg/L); NEO, neomycin (2 mg/L); EDA, edaravone (0.1 mM). Bold indicates statistically significant results ($P < 0.05$).

Table 4. Expression of chromosomal genes related to SOS response in CTX-M-1-producing *E. coli* carrying an IncI1 plasmid when submitted to subinhibitory concentrations of different compounds

	$2^{-\Delta\Delta CT}$						
Gene	C	FLO	FLO + EDA	OTC	OTC + EDA	NEO	EDA
recA	1	**2.93 ± 1.31**	**0.75 ± 0.27**	**2.97 ± 0.92**	3.20 ± 1.32	1.31 ± 0.35	1.57 ± 0.44
sfiA	1	3.40 ± 2.99	0.34 ± 0.35	**11.08 ± 6.23**	11.00 ± 4.14	1.12 ± 0.44	1.12 ± 0.38

FLO, florfenicol (4 mg/L); OTC, oxytetracycline (0.5 mg/L); AMX, amoxicillin (512 mg/L); NEO, neomycin (2 mg/L); EDA, edaravone (0.1 mM). Bold indicates statistically significant results ($P < 0.05$).

together with the other tetracyclines, corresponds to one of the most commonly used antibiotics in food-producing animals in Europe and the USA in 2021.[34,37]

Interestingly, it has previously been shown that the presence of oxytetracycline could enhance an increase in the PCF from *Aeromonas salmonicida* to *E. coli*.[38] Moreover, Holman *et al.*[39] demonstrated an up-regulation of invasion and attachment genes in *Salmonella* spp. after exposure to chlortetracycline and florfenicol. Although the persistence, survival and increased rates of plasmid conjugation after exposure to subinhibitory antibiotic concentrations have been attributed to the SOS response,[40–42] it was suggested that the up-regulation was not related to that system.[39] This hypothesis is opposite to literature results, as well as ours, since we observed a correlation between the SOS active response (*recA* and/or *sfiA* genes) and ROS production with the enhanced effect in conjugation frequencies for both IncL and IncI1 plasmids when exposed to florfenicol and oxytetracycline. In another study, Tang *et al.*[43] evaluated at a molecular level the effect of subdosages of oxytetracycline on antibiotic resistance genes during anaerobic digestion in livestock wastewater influent. In agreement with the data presented here, they showed that a low dose of oxytetracycline increased ROS and SOS responses, contributing to the dissemination of resistance genes by promoting pili and communication responses.[43]

The IncL-type plasmids carrying the OXA-48-encoding gene are globally reported in Enterobacterales, and *Klebsiella pneumoniae* carrying these features are considered one of the most frequent causes of nosocomial infections in humans.[44] OXA-48 was formerly associated with human isolates, but there has been observed an increase in reports of its gene inserted in IncL plasmids in isolates from companion animals[45,46] and the food chain.[47–49] On the other hand, the IncI plasmids are widely reported to carry the CTX-M-1-encoding gene among *E. coli* strains recovered from animals, such as poultry, swine and cattle sources.[12,50,51] The *bla*$_{CTX\text{-}M\text{-}1}$ gene inserted in the IncI plasmids has often been associated with *E. coli* ST10, ST58, ST117 and the widespread international clone ST131,[12,51] these clonal backgrounds being notorious potential sources of exchange of resistance genes between human and animal *E. coli* communities.[12] These are the reasons why we chose both IncL and IncI1 plasmids carrying the resistance genes of utmost importance to perform our experiments, trying to get closer to the One Health concept of connection between human and animal communities. The data provided here demonstrate that subinhibitory dosages of florfenicol and oxytetracycline enhanced PCF for IncL and IncI1 scaffolds in *E. coli*. However, it is important to underscore that the effect of these antibiotics was not tested on different incompatibility family plasmids, actually being a limitation of the present study.

We tested the antioxidant edaravone, a molecule that acts as an ROS free-radical scavenger.[52] This antioxidant molecule is a synthetic pyrazolone derivative and can act by modulating oxidative stress and the production of ROS in bacteria.[53,54] In a recent study we performed, edaravone was capable of significantly reducing the conjugation frequency when added to the treatment of *E. coli* with subinhibitory concentrations of antibiotics.[20] Our results are therefore in accordance with the aforementioned study, once we could observe significant reductions of PCF for florfenicol and oxytetracycline in both IncL and IncI1 plasmids when comparing with those treated only with antibiotics.

Subinhibitory concentrations are especially worth considering in veterinary practice since delivery of antibiotics is often subject to a high level of inconsistency in animal husbandries, for example through food supplementation, which basically generates

variable concentrations of antibiotics and other compounds upon eating. In addition, the environment can be contaminated with residual concentrations of antibiotics. Our data confirm that the use of subinhibitory dosages of florfenicol or oxytetracycline significantly enhance PCF for IncL and IncI1 plasmid scaffolds in *E. coli*. We could demonstrate that edaravone had a significant impact on reducing PCF, and may be considered for a combined antimicrobial therapy. The food chain is a crucial transmission route for antimicrobial resistance between animal and human populations. The overuse, suboptimal use and routine use of antibiotics in food-animal farms may potentially increase the risk of zoonotic transmission of relevant β-lactamase-encoding genes for human medicine, as well as of other genes conferring resistance to critically important antimicrobials in public health.

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Transparency declarations

None to declare.

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