

## INDUCTION OF RESISTANT MUTANTS OF *SALMONELLA* *TYPHIMURIUM* UNDER ENROFLOXACIN AND NATURAL ALTERNATIVES FOR CONTROL IN PIGS

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### Introduction

The increase in bacterial resistance to Enrofloxacin (ENR) in recent years has been associated with the selective growth of single-step mutant strains resistant to the frequent dose in use, based on the Minimum Inhibitory Concentration (MIC). Because of that, it is supported the need to establish optimal dosage intervals taking into account the minimum concentration of antibiotic capable of inhibiting the growth of pre-existing or first pass mutant strains: Mutant Prevention Concentration (MPC). Therefore, it is of importance to maintain the antimicrobial and molecular monitoring of quinolones and to search for new alternatives, such as the use of essential oils for the control of salmonellosis.

The objectives of the present study were to induce the emergence of mutant strains of *S. Typhimurium* with reduced sensitivity to ENR, molecular characterization of the Quinolone Resistance Determining Region (QRDR) and to determine the MPC of ENR. Moreover, in order to examine the molecular basis of resistance to this fluoroquinolone, single-step induced strains selected from sub-MPC plaques for point mutations in the QRDR of the *gyrA*, *gyrB*, *parC* and *parE* genes were investigated.

In addition, the antimicrobial activity of EOs of cinnamon, oregano, red thyme and common thyme was evaluated against the strains obtained after being overexposed to ENR.

### Material and methods

**Bacterial strains:** Two clinical strains susceptible to ENR of *S. Typhimurium*, isolated from clinical cases of pigs, and the reference strain ATCC 14028 were subjected to study.

**Antibiotic:** Enrofloxacin (HPLC 98% purity) of Sigma-Aldrich Co. (San Luis, Missouri, USA) has been used for this study.

**Essential oils:** Cinnamon (*Cinnamomum zeylanicum*) (cinnamaldehyde, linalool, eugenol); oregano (*Origanum vulgare*) (carvacrol, thymol,  $\gamma$ -terpinene); common thyme (*Thymus vulgaris*) (Thymol, *p*-cymene, linalool) and red thyme (*Thymus zygis*) (Thymol, *p*-cymene,  $\gamma$ -terpinene, linalool) were utilized, all of them of natural origin and provided by Aromium S.L., Barcelona-Spain.

**Enrofloxacin susceptibility test:** The methods of microbroth dilution and agar dilution were used to determine the MIC of ENR, according to CLSI guidelines (CLSI, 2015). Microdilution method was performed in microtiter plates, containing twofold dilutions of ENR. The agar dilution method consisted in incorporating the ENR into the

agar, containing each plate different concentrations of ENR in double dilution together with the bacterial inoculum until reach a final concentration of  $5 \times 10^5$  CFU/mL.

**Enrofloxacin resistance-inducing test and determination of MPC:** To establish MPC, a bacterial inoculum of  $\geq 10^{10}$  CFU/mL was used. This inoculum was cultured for 5 days on Müller Hinton agar plates supplemented with ENR (1x to 64x fold MIC) to identify the mutant strains and to determine the MPC. From plates supplemented with an enrofloxacin concentration  $\geq 1x$  MIC, possible single-step mutants were randomly selected, cultured on enrofloxacin free agar plates for three serial passage, serotyped and then stored at  $-70$  °C.

**DNA sequencing of QRDR:** In order to examine the molecular basis of resistance to this fluoroquinolone, single-step induced strains selected from sub-MPC plaques for point mutations in the resistance-determining region to quinolones (QRDR) of the *gyrA*, *gyrB*, *parC* and *parE* genes were investigated.

**Susceptibility test of EOs:** The original strains and a selection of the strains obtained after induction were put together with the EOs by means of microdilution technique. This step was performed in microtiter plates, containing twofold dilutions of each EO tested and a bacterial inoculum with a final concentration of  $5 \times 10^5$  CFU/mL. The MIC of each test was determined as the lowest concentration of EO that prevents the visible growth of the bacteria in wells.

## Results

The three strains of *S. Typhimurium* used in this study showed a MIC of 0.0625 µg/mL in test of microbroth dilution and agar dilution. This way, the concentration of EOs necessary to prevent the total growth of the bacterial population was in the three cases 4 times the MIC (MPC = 0.25 µg/mL).

In all assays, strains with reduced susceptibility to enrofloxacin (MPC/MIC = 4) were obtained, although none of them reached the point of resistance.

Characterization of QRDR region of the original strains and the strains obtained in the induction showed the wild type (*wt*) of the genes *gyrA*, *gyrB*, *parC* and *parE*. However, none of them presented genetic mutations.

The EOs of cinnamon, oregano, red thyme and common thyme demonstrated a strong antimicrobial activity against *S. Typhimurium* strains with reduced susceptibility to enrofloxacin (MIC 312.5 - 625 µg/mL).

## Conclusions

The reduction observed in the susceptibility to ENR of the tested strains, without isolated mutations in the target genes of the QRDR region, supports the importance of other mechanisms described for the development of quinolone resistance (alterations in external membrane and the active efflux pump).

The selective growth of strains with reduced susceptibility to concentrations of ENR higher than MIC, highlights the importance of MPC in the determination of dosage regimens.

The results of this research show that EOs of cinnamon, oregano, common thyme and red thyme are able to control the growth of strains with reduced susceptibility to ENR arising by exposure to sub-MPC doses. These AE and/or their combination with

ENR would prevent the selective growth of resistant bacterial subpopulation and, in consequence, therapeutic failure.

## **References**

[CLSI] Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 3rd ed. CLSI supplement VET01S. Wayne, PA 2015; 3:14-117.