Comparison of the *in vitro* neutralizing effects of amoxicillin and colistin used separately or in combination, and ciprofloxacin, on *E. coli* endotoxin (lipopolysaccharide: LPS)

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Introduction

Escherichia coli is considered as the first bacteria responsible for economic losses on young cattle. It affects both the dairy and meat sector (1). *E. coli* infections lead to diarrheas and septicemia.

The LPS (endotoxin), a component of the external membranes of Gram- bacteria, is one of the most important virulent factors of these bacteria. LPS is involved in local and systemic inflammations during *E. coli* infections mainly by inducing pro-inflammatory cytokines (TNFα, IL1, IL6, …) possibly responsible of septic shock (2).

During antibiotic treatments, lysed bacteria may release a high quantity of endotoxins increasing the severity of the cattle clinical status. Therefore the goal of the medical treatment should not only be to stop the infection but also to neutralize toxins liberated by the lysis of targeted bacteria.

Objectives

The objective of this *in vitro* study was to compare the neutralization effect on the toxic action of LPS, *E. coli* toxin, of several antibiotics commonly used against this bacteria. For that purpose, colistin (Co), amoxicillin (A), used separately and in association (Co/A) and ciprofloxacin (Ci), active metabolite of enrofloxacin (En) were tested.

Materials & Methods

Concentrations of the different antibiotics used in this study are based on the pharmacokinetics data provided by manufacturers, Virbac for the association Co/A (Potencil®), Bayer for En and Ci (Baytril®). For each antibiotic, the expected maximal concentrations (Cmax) during administration on cattle for daily treatment during 3 days were tested. Values for A and Co are coming from internal data, whereas for Ci (active metabolite of En), values are coming from Baytril® 10% injectable SPC (Bayer) and from a scientific publication (3). Due to the toxicity of DMSO (used as En solvent) against cell cultures, En was excluded from experiments and only Ci results are presented.

The A549 cell line (human cells of ATCC CCL-185 epithelial pulmonary carcinoma) was chosen to be exposed to LPS from *E. coli* O111/B4 and to antibiotics. These cells are sensitive to *E. coli* enteropathogenic strains (EPEC) and respond to LPS toxicity (4).

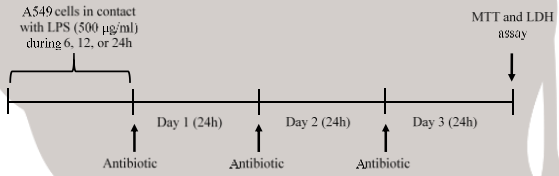
After several tests, LPS of 500µg/ml concentration was kept, as it mimics a strong endotoxemia.

Proportion of viable cells (%VC) after contact with LPS was measured by 2 methods:

- LDH method: detecting the release of lactate dehydrogenase by damaged cells (Cytotoxic Detection Kit, Sigma-Aldrich).
- MTT method (Tetrazolium dye): measuring the mitochondrial activity by colorimetry.

Control cells were cultured only in standard medium.

To mimic a real infection and its treatment, A549 cells were firstly incubated with 500 µg/ml LPS for 6-24 h ('bacterial infection'), and then treated with antibiotics (at concentration indicated in Figures 1 & 2) according to the following protocol:



Results and Discussion

Preliminary *in vitro* studies confirmed the safety of all used antibiotics.

In experimental conditions mimicking a real infection (Figures 1 & 2), the most critical factor for cells viability was the exposition time to LPS before adding the antibiotic. After 6h of contact between cells and LPS, all the tested antibiotics demonstrated a comparable ability to neutralize LPS toxicity. %VC were close to 80%-90% in all antimicrobial-treated cultures, and lower than 20% in controls. For cells exposed up to 12h, Co/A treatment leads to significantly (P<0.001) higher %VC than treatment with Ci which has lost its ability to neutralize endotoxin. However %VC were similar in Co/A, A, or Co groups (approx. 60%). For cells exposed up to 24h, %VC were generally low (25% up to 40% for Co/A, 0% up to 20% for A, Co and Ci), Co/A-treated cultures yielding %VC significantly higher (P≤0.01 or P≤0.05) than cultures treated with any of the other antimicrobials.

In our *in vitro* study, the Co/A association demonstrated a stronger ability to neutralize LPS compared to its components used separately or Ci.

In the field, due to the delay between the moment of infection, the incubation period and the detection of clinical signs by farmers, treatments are often postponed. As contact time of cells with LPS is critical, this experimentation demonstrates that it is key to treat as early as possible.

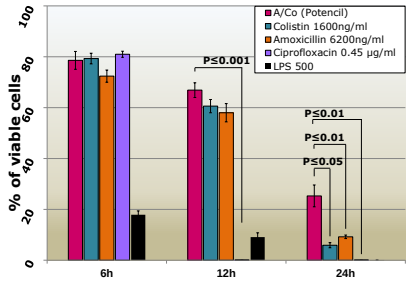


Fig. 1: Comparison of the neutralizing effect of 500µg/ml of LPS by different antibiotics or association of antibiotics (LDH method)

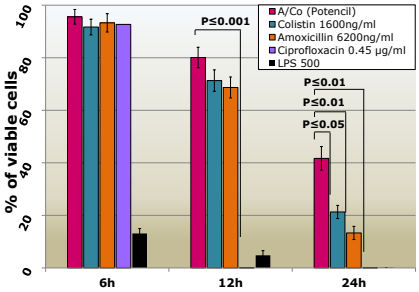


Fig. 2: Comparison of the neutralizing effect of 500µg/ml of LPS by different antibiotics or association of antibiotics (MTT method)

Conclusion

This *in vitro* study demonstrated the ability of antibiotics, used in concentrations detected in plasma of treated animals, to neutralize LPS (*Escherichia coli* endotoxin) toxic effect in an experiment mimicking natural infections and treatments. This experimentation also proved the significant greater effect of the colistin/amoxicillin combination, even after 24h, compared to colistin and amoxicillin used separately, or a fluoroquinolone, ciprofloxacin. The delay between the initial symptoms and the antibiotic treatment administration is also critical to fight against infection and to fasten recovery. In case of infection, endotoxins concentration is increasing due to bacterial multiplication and bacterial lysis caused by the bactericidal effect of the treatment. To minimize the negative effects linked to toxins release, antibiotic treatments should also neutralize these toxins.

This study demonstrated that the colistin/amoxicillin association has not only the ability to fight against bacteria but also presents a great interest in case of endotoxemia in animals.

(1) R. Kolenda et al., 2015. Front. Cell. Infect. Microbiol, 5, 23. (2) D. Angus et al., 2013. N. Engl. J. Med, 369, 840-851. (3) Q. McKellar et al., 1999. Am. Soc. Microbiol, 43, 1988-1992. (4) K. Nishio et al., 2013. Redox Biol.1 :97-103