Tildipirosin: An effective antibiotic against Glaesserella parasuis from an in vitro analysis

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ABSTRACT

Tildipirosin is a latest generation macrolide that is used to battle infection diseases caused by Gram-negative bacteria. Recent studies have shown the effectiveness of this antimicrobial agent against *Actinobacillus pleuropneumoniae*; however, little information is available about *Glaesserella parasuis*, the etiological agent of Glässer's disease. In this study, the Tildipirosin activity to 100 Brazilian clinical isolates of *G. parasuis* was assessed using a broth microdilution assay. A total of 90% of *G. parasuis* isolates were sensitive at concentrations ≤ 4 μg/mL Tildipirosin, thus showing to be efficiently controlled by the therapeutic concentration recommended for pigs. On the other hand, a total of ten isolates have shown resistance to this antibiotic, with a minimal inhibitory concentration (MIC) ≥ 8 and ≤ 16 μg/ml. Notably, our findings highly support the use of Tildipirosin for treating Glässer's disease outbreaks, and it also advises the using of MIC approach to monitor the evolution of sensitivity or resistance exhibited by *G. parasuis* to this molecule, as well as to adjust therapeutic doses when necessary.

Glässer's disease (GD) is a systemic inflammatory infection that affects young pigs, mainly in nursery phase, causing significant economic losses for the pig industry due to the mortality of infected animals and the expenses derived from the use of antimicrobial agents (Guizzo et al., 2018). GD is a bacterial disease caused by *G. parasuis*, a Gram-negative microorganism able to colonize effectively the porcine respiratory tract during the first week of life (Cerda-Cuellar et al., 2010).

GD prevention is mainly carried out by means of vaccination using monovalent or bivalent inactivated whole-cell based vaccines (Guizzo et al., 2018). Although these vaccines are formulated using *G. parasuis* serovars with a high prevalence and worldwide distribution, they are only capable to induce a robust antibody response against the homologue's polysaccharide capsule, converting the broad-spectrum protection against most of the GD causative *G. parasuis* serovars (SV) limited or even absent.

Recently, we have demonstrated that the commercially available vaccines in Brazil do not include the most prevalent SVs isolated from pigs suffering GD and that a substantial proportion of disease was caused by non-typeable (NT) strains that can likely represent at least

nine new serovars (Espíndola et al., 2019). This situation can easily explain why the veterinarians are still using massive amounts of antimicrobial molecules in animal feed to control the clinical presentation of GD during the nursery phase (Brazilian perspective, personal observation). In parallel, due to the continuous cases of GD, even in farms using commercial vaccines (possible infected with different serovars than those included in the vaccine), the production of autogenous vaccines emerges as a rational strategy to control the infection in the short-term.

As a consequence of the massive use of antibiotics in pig production, antimicrobial resistance becomes a serious threat to animal and public health (Prestinaci et al., 2015). Although *G. parasuis* can be sensitive to a wide range of molecules classes, macrolides such as Tylosin and Tilmicosin are frequently used to control GD outbreaks (Dayao et al., 2014), and their action consist in the inhibition of the bacterial protein synthesis (Chen et al., 2010). As an evolutionary mechanism, microorganism can also develop cross-resistances between macrolides and lincosamides (Roberts, 2008), possibly by plasmids carrying lnu(C) inactivanting gene of Lincosamyn (Chen et al., 2010), raising a warning

light on these two antimicrobial classes. In a previous study, we demonstrated a concern about the resistance trend of G. parasuis field strains to Tylosin Miani et al., 2017. Here, we evaluated the in vitro efficacy of Tildipirosin, a semi-synthetic derivative of the naturally occurring 16-membered macrolide Tylosin on clinical isolates of *G. parasuis*.

A total of 100 clinical isolates of *G. parasuis* selected from the bacterial collection kept on the Laboratory of Microbiology and Advanced Immunology at the University of Passo Fundo/Brazil, were tested in this investigation. The strains were isolated from January 2013 to June 2018 from pigs with clinical signs and macroscopic lesions consistent with GD. Amongst the isolates, 58 were from systemic sites of infection (joints, peritoneum, pericardium and brain) and 42 were from lungs. Each of these isolates were from different farms and states with high pig production rates. In addition, after culture isolation, all samples were molecularly typified (Espíndola et al., 2019). The susceptibility test was carried out by the microdilution assay (96 well plates, TPP, Swiss) according to the recommendations of the Clinical and Laboratory Standard Institute (2013) for *A. pleuropneumoniae*. Tildipirosin pure powder was supplied by Merck Sharp & Dhome (Germany) and was kept under refrigeration (4–8ºC). Prior to the test, a fresh working stock solution at 2 mg/mL of Tildipirosin was prepared using double distillated water. The culture medium was Pleuropneumonia-Like Organisms (PPLO) broth supplemented with 75 μg/mL nicotinamide adenine dinucleotide and 2,5 mg/mL glucose (Sigma-Aldrich, USA). The 96-well plates were prepared immediately before use and different Tildipirosin concentrations were added until obtaining serial double dilutions ranging from 0,032 to 256 μg/mL. All tests were carried out in duplicate. In each plate, two wells without Tildipirosin were used as positive control of *G. parasuis* growth, and two wells without bacteria were used as a negative control of *G. parasuis* growth. These same controls were used to test *A. pleuropneumoniae* ATCC 2709 reference strain, which was used to validate each plate tested in this study. Then, *G. parasuis* (5×10^4 organisms quantified by flow cytometry as described by Barasuol et al. (Barasuol et al., 2017) was added to each well and incubated at 37ºC and 5% CO_2 for 24 h, as previously described (Miani et al., 2017). The minimal inhibitory concentration (MIC) was defined as the lowest Tildipirosin concentration able to inhibit *G. parasuis* growth. The cut off value was adjusted to 4 μg/mL, taking into consideration the peak of tildipirosin concentration (C_{max}) observed in pig lungs after intramuscular injection of 4 mg/Kg of Tildipirosin Lei et al., 2018 . The MIC ranges were analyzed using non-parametric Mann-Whitney Test where a P-value of ≤ 0.05 was considered as a significant difference.

The results of the antimicrobial activity tests are shown in Fig. 1. The MIC distribution of Tildipirosin against *G. parasuis* [SV1, $n = 5$; SV4, $n = 31$; SV5, $n = 19$; SV7, $n = 6$; SV12, $n = 11$; SV13, $n = 3$; SV14, $n = 9$; SV15, $n = 3$; Non-typeable (NT) group α, $n = 6$; NT group γ, $n = 7$) varied from 0.03 to 16 μg/mL, with three peak values, observed at 0.03, 0.06 and 0.25 μg/mL. The distribution ratio of the 3 mean peaks of MIC were 37% for 0.03 μg/mL, 15% for 0.06 μg/mL and 14% for 0.25 μg/mL (Fig. 1A). The overall MIC_{50} and MIC_{90} were 0.06 and 4 μg/mL, respectively. Taken into consideration the sample sources, systemic isolates were significantly ($P < 0.05$) more sensitive to Tildipirosin than the pulmonary ones; they showed MICs values lower than those isolates recovered from lung site (MIC_{50} = 0.06 vs 0.25 μg/mL and MIC_{90} = 0.5 vs 8 μg/mL, respectively) (Fig. 1B). These differences were not expected since macrolides, especially Tildipirosin, have an extraordinary ability to accumulate in different compartments of lung tissue and to persist in fluids surrounding the lung epithelial cells (Berlin et al., 2017; Menge et al., 2012; Rose et al., 2013;Villarino et al., 2013).

When the distribution of susceptibility profile of clinical isolates was analyzed in comparison to year of recovery of *G. parasuis*, it was observed that all isolates with a MIC higher than 4 μg/mL (10%) were isolated from 2015 forward, given the epidemiological cut-off (ECO) reported by Lei et al., 2018, of note, only one year after Tildipirosin introduction into Brazilian pig farms.

The correlation between the highest Tildipirosin antimicrobial concentration in serum (C_{max}) and the *in vitro* proposed MIC is currently used as a measure to determine the *in vivo* effectiveness of a treatment. In this stage, the administration of the therapeutic concentration of Tildipirosin by the intramuscular route in pig (4 mg/kg) would render a lung C_{max} of 4.06 $\pm$ 0.65 μg/mL at 5.33 $\pm$ 2.37 h after injection (Lei et al., 2018). Taking into consideration this value, 90% of clinical isolates tested in this study showed a MIC $\leq$ 4 μg/mL, so that they could be effectively treated with the commercially available presentation of Tildipirosin, using the therapeutic dose recommended by the manufacturer.

Unfortunately, the historical data of use of Tildipirosin in farms from which *G. parasuis* clinical isolates were recovered is not available and, therefore, the establishment of a correlation between MIC values and the use of this macrolide is not possible. According to Fig. 1, a total of ten clinical isolates showed MICs $\geq$ 8 ($n = 5$) and $\leq$ 16 μg/mL ($n = 5$), values far above those of ECO, thus suggesting already the circulation of resistant strains to Tildipirosin, as reported in China (Lei et al., 2018).
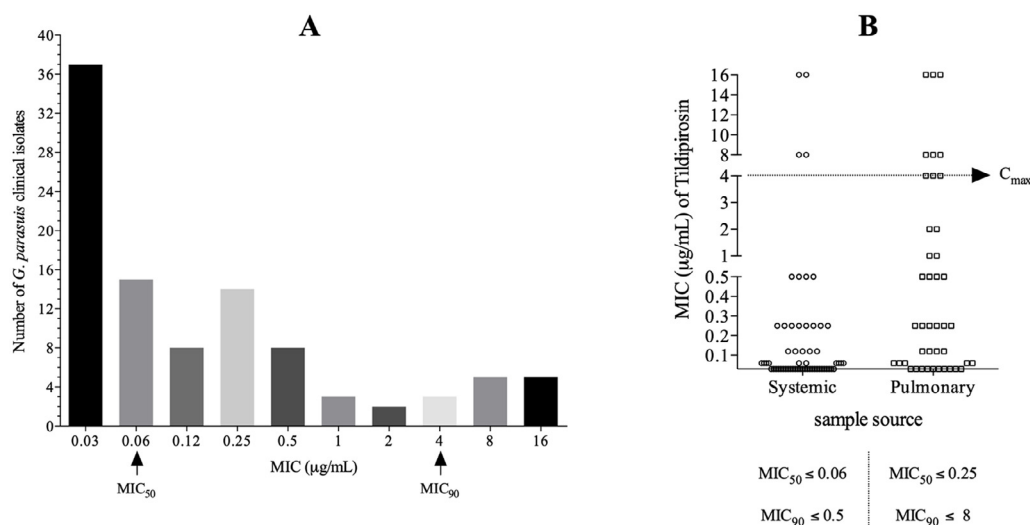

Fig. 1. MIC distributions of Tildipirosin against *Glaesserella parasuis* clinical isolates. A: Cumulative isolates per MIC and values of MIC_{50} and MIC_{90}. B: MIC distributions for systemic and pulmonary isolates. C_{max} is indicated by dotted arrow.

The mechanism of action of Tildipirosin consists of the inhibition of the bacterial protein synthesis as a consequence of binding to 23S ribosomal RNA for 50S subunit (Chen et al., 2010). As an adaptive mechanism, lincosamides develop a resistance mechanism based on the alteration of the target site of action of the antibiotic, resulting in cross-resistances among macrolides or even among lincosamides and streptogramin B (MLSB) (Roberts, 2008). In this same line, Aller-Moran—Aller-Moran et al. (2015) showed cross-resistance between Tiamulin and Valnemulin, observed as well between Lincomycin and Tylosin Pringle et al., 2004 . These evidences need to be carefully interpreted in our particular case; in 2017 we demonstrated the circulation of *G. parasuis* clinical isolates highly resistant to Lincomycin, Tiamulin and Tylosin Miani et al., 2017. Although the cross-resistance between macrolides would be a logical explanation for understanding the presence of resistant strains to Tildipirosin in Brazil, further studies need to be done to investigate the evolutionary process of *G. parasuis* resistance to Tildipirosin in regions with high resistance to Lincomycin. In addition, Chen et al., 2010 evidenced the presence of the lnu(C) inactivating gene of Lincosamyn in extrachromosomal plasmids isolated from clinical strains of *G. parasuis*. This observation contributes to evolutionary understanding of how Lincomycin resistance can be spread between or through Gram-negative bacterial species.

Finally, macrolides are one of few available therapies for serious campylobacter infections in human, particularly in children, in whom quinolones are not recommended for treatment (World Health, 2011). Due to this, WHO list of critically important antimicrobials include macrolides in the top priority group of molecules that should be avoided their use in food-producing animals (World Health, 2017); however, when there is no other possibility, the use of these molecules need to be rational. In this line, contrary to Erythromycin, Tylmicosin and Tylosin which have been used for a long time as growth promoters (banned in several countries), Tildipirosin is only available for parenteral application, restricting their use for therapeutic application, which rationally mitigates its use for prophylaxis or metaphylactic purposes.

In conclusion, our results show that Tildipirosin is recommended for treating clinical cases of GD. Due to the existence of clinical isolates resistant to this antimicrobial agent, MIC test must be used periodically in order (i) to establish the therapeutic dose, and (ii) to monitor the evolution of *G. parasuis* resistance to Tildipirosin.

Declaration Competing of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CF & MCS are employees of MSD, the company that commercializes the Tildipirosin. MSD has not influenced the writing of this article. The remain authors report no financial or personal interests with other persons and public or private organizations that can influence the results reported in this article.

Acknowledgements

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