Veterinary Microbiology 134 (2009) 305–310

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

In vitro antimicrobial activity against 10 North American and European *Lawsonia intracellularis* isolates

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ARTICLE INFO

Article history:
Received 26 June 2008
Received in revised form 7 August 2008
Accepted 14 August 2008

Keywords:
Proliferative enteropathy
Lawsonia intracellularis
MIC
Antimicrobial activity

ABSTRACT

The objective of this study was to determine the *in vitro* minimum inhibitory concentration (MIC) of antimicrobials against 10 isolates of *Lawsonia intracellularis*, the etiological agent of proliferative enteropathy (PE). Antimicrobials tested included carbadox, chlortetracycline, lincomycin, tiamulin, tylosin and valnemulin. The MIC of each antimicrobial against *L. intracellularis* was determined using a tissue culture system and was identified as the lowest concentration that inhibited 99% of *L. intracellularis* growth, as compared to the antimicrobial-free control. Each antimicrobial concentration was evaluated for both intracellular and extracellular activity against *L. intracellularis*, an obligately intracellular bacterium. When tested for intracellular activity, carbadox, tiamulin, and valnemulin were the most active antimicrobials with MICs of $\leq$0.5 μg/ml. Tylosin (MICs ranging from 0.25 to 32 μg/ml) and chlortetracycline (MICs ranging from 0.125 to 64 μg/ml) showed intermediate activities and lincomycin (MICs ranging from 8 to >128 μg/ml) showed the least activity. When tested for extracellular activity, valnemulin (MICs ranging from 0.125 to 4 μg/ml) was the most active against most *L. intracellularis* isolates. Chlortetracycline (MICs ranging from 16 to 64 μg/ml), tylosin (MICs ranging from 1 to >128 μg/ml), and tiamulin (MICs ranging from 1 to 32 μg/ml) showed intermediate activities. Lincomycin (MICs ranging from 32 to >128 μg/ml) showed the least activity. Our *in vitro* results showed that each *L. intracellularis* isolate had a different antimicrobial sensitivity pattern and these data can be utilized as an *in vitro* guideline for the further antimicrobial evaluation of field *L. intracellularis* isolates.

1. Introduction

Proliferative enteropathy (PE) is one of the most prevalent enteric bacterial diseases in grower and finisher pigs. The etiological agent of this disease is an obligate intracellular, Gram-negative bacterium named *Lawsonia intracellularis*. The treatment of a PE outbreak on a pig farm often involves antimicrobial therapy. However, since little information is available on *in vitro* antimicrobial sensitivities against *L. intracellularis* infection, the selection of an appropriate antimicrobial is difficult. The paucity of information is due to the fact that standard antimicrobial assays are not applicable to evaluate the antimicrobial activities of most intracellular organisms since these bacteria only propagate themselves inside the host cell. Therefore, most *in vitro* studies of antimicrobial activities against obligate intracellular bacteria are undertaken through a complicated cell culture system (McOrist et al., 1995b; Gnarpe et al., 1996; Ives et al., 2000; Horowitz et al., 2001). Furthermore, few strains of *L. intracellularis* have been successfully isolated and maintained *in vitro*. Of these, only three European isolates have been tested *in*

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doi:10.1016/j.vetmic.2008.08.007

vitro for antimicrobial susceptibilities using a tissue culture system (McOrist and Gebhart, 1995; McOrist et al., 1995b).

It has been a decade since these antimicrobial activity studies of *L. intracellularis* have been reported, and no further *in vitro* studies have been published to update or expand upon the limited data existing for the antibiotic sensitivity of *L. intracellularis*. Therefore, the objective of this study was to determine the *in vitro* antimicrobial sensitivities of 10 isolates of *L. intracellularis* obtained from both North America and Europe against six antimicrobial compounds that have been used for the treatment and control of PE in pigs.

2. Materials and methods

2.1. Source and preparation of antimicrobials

The following antimicrobial agents were purchased as pure chemicals: carbadox, chlortetracycline hydrochloride, lincomycin hydrochloride and tylosin tartrate (Sigma–Aldrich, Missouri, United States). Tiamulin hydrogen fumarate and valnemulin hydrochloride were supplied as pure chemicals from Novartis Animal Health (Basel, Switzerland). The stock solutions of all antimicrobial compounds were prepared to a final concentration of 2560 μg/ml. Each antimicrobial solution was sterilized by filtration using 0.2 μm-pore size filters. The stock solution of carbadox was first dissolved with 0.1N NaOH and then was diluted in sterile distilled de-ionized water. The stock solutions of the other compounds were dissolved directly in sterile distilled de-ionized water and all were kept at −20 °C until use. Once the antimicrobials were thawed, they were used and kept refrigerated for up to 3 days. A series of two-fold dilutions were made from the stock solutions, and these were then diluted 1:10 with culture medium to resultant final concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg/ml. Each concentration of antimicrobial was tested in triplicate.

2.2. Bacterial strains and preparation

A total of 10 *L. intracellularis* field strains collected between 1983 and 2006 from infected pigs from North America and Europe were tested. Six *L. intracellularis* strains used were from North America: PHE/MN1-00, VPB4, KKumn04, NWumn05, DBumn06 and 47216-06. Three *L. intracellularis* strains used were from the United Kingdom: LR189/5/83, 963/93 and 916/91; and one *L. intracellularis* strain used was from Denmark: D15540. All strains were stored at −72 °C until use.

All strains of *L. intracellularis* were grown in murine fibroblast-like McCoy cells (CRL 1696, American Type Culture Collection, Virginia, United States) and were maintained in a cell culture system as described previously (Guedes and Gebhart, 2003; Wattanaphansak et al., 2005).

2.3. Antimicrobial sensitivity testing

A tissue culture system was modified from a previous study (McOrist et al., 1995b) to determine the minimum inhibitory concentration (MIC) of each antimicrobial against *L. intracellularis*. Briefly, the frozen bacteria were thawed and grown in cell culture for at least 3 continuous passages to achieve 100% infection of the McCoy cell monolayer. All *L. intracellularis* isolates were tested twice and each replicate was prepared independently. Each strain of *L. intracellularis* was harvested from the monolayer as described earlier (Guedes and Gebhart, 2003; Wattanaphansak et al., 2005), diluted with 100 ml culture medium, and 100 μl of this bacterial suspension was inoculated onto one-day-old McCoy cells in 96-well tissue culture plates (Nalge Nunc International, New York, United States).

In this study, the MICs were expressed for both intracellular and extracellular activities. Intracellular MIC testing was conducted in order to measure the effect of antimicrobials on *L. intracellularis* after the bacteria had infected the enterocytes. For intracellular testing, a previously published assay (McOrist et al., 1995b) was used with minor changes to the cell line and the bacterial concentration. Briefly, 100 μl of bacterial suspension containing approximately 10^6–10^7 *L. intracellularis* organisms/ml, a quantification method described by Guedes and Gebhart (2003), was inoculated onto one-day-old McCoy cells 24 h before exposure to the antimicrobials. This permitted sufficient time for *L. intracellularis* to penetrate the host cells prior to antimicrobial treatment. After incubation, the bacterial suspension was removed and replaced with 100 μl of fresh culture medium containing various concentrations of antimicrobials at 1, 2, and 3 days post inoculation, followed by fresh culture media on day 4 with no antimicrobial as previously described (McOrist et al., 1995b).

The extracellular MIC testing was designed to mimic the effect of antimicrobial on *L. intracellularis* when the bacterium is free in the gut lumen before infecting the intestinal cells. For extracellular testing, we followed a previously described approach (McOrist et al., 1995b) with minor changes. Briefly, a series of two-fold dilutions of stock antimicrobials were added to culture medium containing *L. intracellularis*. The suspension was incubated at 37 °C in 8.0% oxygen, 8.8% carbon dioxide, and 83.2% nitrogen atmosphere for 2 h without mixing, allowing direct exposure of the bacteria to the antimicrobials. After incubation, 100 μl of the bacterial suspension was transferred to infect one-day-old McCoy cells. The medium was removed after 24 h incubation and replaced with 100 μl of new culture medium without any antimicrobials for 3 consecutive days. Following the media removal each day, the infected plates were exposed to hydrogen gas and the plates were then kept at 37 °C for 5 days in an incubator with 8.0% oxygen, 8.8% carbon dioxide and 83.2% nitrogen as the atmosphere.

After 5 days incubation, supernatant from the infected plates was removed and the cell culture monolayer was fixed with 100 μl of cold 50% acetone and 50% methanol for 1 min. To assess the inhibitory effect of each antimicrobial on *L. intracellularis* proliferation, the infected plates were stained using a modified immunoperoxidase monolayer assay staining method (Guedes et al., 2002) with primary antibody from a rabbit hyperimmunized