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

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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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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

Table 1
Summary of intracellular and extracellular MIC endpoints for six antimicrobial agents against 10 *L. intracellularis* isolates, six obtained from North America and four from Europe, measured by using tissue culture system with 5 days of incubation

Strains of LI	Country of origin	Year	No. of passage	Antimicrobial agents (μg/ml)											
				Carbadox		Chlortetracycline		Tylosin		Lincomycin		Tiamulin		Valnemulin	
				Intra MIC[a]	Extra MIC[b]	Intra MIC	Extra MIC	Intra MIC	Extra MIC	Intra MIC	Extra MIC	Intra MIC	Extra MIC	Intra MIC	Extra MIC
PHE/MN1-00	USA	2000	169	0.125	16	8	64	8	64	>128	>128	0.125	4	0.125	0.25
			170	0.25	16	4	32	2	64	>128	>128	0.125	8	0.125	0.25
VPB4	USA	1991	165	0.25	32	4	64	8	128	>128	>128	0.125	32	0.125	2
			166	0.25	32	16	64	32	128	>128	>128	0.5	8	0.125	0.25
KKumn04	USA	2004	17	0.125	4	32	32	0.5	1	16	>128	0.125	1	0.125	0.125
			18	0.125	4	16	64	0.25	1	16	>128	0.125	1	0.125	0.125
NWumn05	USA	2005	21	0.125	16	64	64	8	>128	>128	>128	0.125	16	0.125	4
			22	0.125	8	64	64	4	128	>128	>128	0.125	8	0.125	1
DBumn06	USA	2006	8	0.125	4	0.125	32	4	128	>128	>128	0.125	4	0.125	0.25
			9	0.125	4	0.125	32	4	128	>128	>128	0.125	8	0.125	0.25
47216-06	USA	2006	7	0.125	8	64	64	2	64	>128	>128	0.125	8	0.125	0.5
			8	0.125	8	64	64	2	64	>128	>128	0.125	4	0.125	0.5
D15540	Den	1998	19	0.125	4	0.25	64	1	4	32	>128	0.125	4	0.125	0.125
			20	0.125	4	0.25	32	0.5	2	16	128	0.125	2	0.125	0.125
LR189/5/83	UK	1983	14	0.125	1	0.5	64	1	16	16	>128	0.125	4	0.125	0.125
			15	0.125	1	0.5	64	1	16	16	>128	0.125	4	0.125	0.125
963/93	UK	1993	35	0.125	1	16	32	1	4	8	64	0.125	2	0.125	0.25
			36	0.125	1	8	32	1	4	8	64	0.125	2	0.125	0.25
916/91	UK	1991	16	0.125	1	8	64	2	4	64	64	0.125	2	0.125	0.125
			17	0.125	1	2	16	0.5	2	8	32	0.125	1	0.125	0.125

Each strain of *L. intracellularis* was tested twice and the bacteria were prepared independently for each replicate. USA: the United States of America; Den: Denmark; UK: United Kingdom.
[a] The intracellular MIC.
[b] Extracellular MIC was defined as the lowest antimicrobial concentration that inhibited 99% of *L. intracellularis* proliferation, compared to antimicrobial-free control.

with *L. intracellularis* antigen. The *L. intracellularis* proliferation was evaluated by counting the number of heavily infected cells (HIC) (McOrist et al., 1995b) in each well using an inverted microscope (Olympus, Tokyo, Japan) with a 20× objective lens. Cells were considered to be HIC if the number of intracellular *L. intracellularis* had proliferated to greater than 30 bacteria per cell. The number of HICs in each well was then expressed as a percentage compared to the average HIC of the control wells. The intracellular and extracellular MIC endpoints for each antimicrobial in this study were defined as the lowest antimicrobial concentration that inhibited 99% of *L. intracellularis* proliferation in the McCoy cells after 5 days of incubation (McOrist et al., 1995b). These inhibitions were indicated by the percentage of HIC of each antimicrobial concentration as compared to the antimicrobial-free control.

2.4. Data analysis

The MIC endpoints of each antimicrobial were determined using the median value from a set of triplicate wells. MIC assays were performed in duplicate from independent bacterial preparations, and the duplicate MIC endpoints were expressed for each antimicrobial for each isolate. When the percentage of HIC of *L. intracellularis* in the antimicrobial-free control was less than 50%, the MIC tests for that *L. intracellularis* strain were repeated.

3. Results

The intracellular and extracellular MIC values for antimicrobials used against the 10 *L. intracellularis* isolates in the present study are shown in Table 1. The concentrations of *L. intracellularis* inocula were between 1.2×10^6 and 3.4×10^7 *L. intracellularis* organisms/ml, and each isolate had a range of less than one log between the two replicates. For *L. intracellularis* isolates from North America ($n = 6$), the intracellular activity results showed that carbadox, tiamulin, and valnemulin displayed the highest activity with MICs from $\leq$0.5 μg/ml. Chlortetracycline and tylosin showed moderate activity against *L. intracellularis* with MIC ranges from 0.125 to 64 μg/ml and 0.25 to 32 μg/ml, respectively. Lincomycin showed the lowest activity against most *L. intracellularis* isolates with an MIC range from 16 to >128 μg/ml. The extracellular activity results showed that only valnemulin had high activity against *L. intracellularis* with MICs ranging from 0.125 to 4 μg/ml. Antibiotics with moderate activities against *L. intracellularis* included carbadox with an MIC range from 4 to 32 μg/ml, chlortetracycline with an MIC range from 32 to 64 μg/ml, tiamulin with an MIC range from 1 to 32 μg/ml, and tylosin with an MIC range from 1 to >128 μg/ml. All *L. intracellularis* isolates from North America had the lowest extracellular activity to lincomycin with MICs from >128 μg/ml.

MIC results for the European isolates ($n = 4$) were similar to the North American isolates in that, for the intracellular MICs, carbadox, tiamulin, and valnemulin had the highest activity against *L. intracellularis* with MICs of 0.125 μg/ml. Antimicrobials with moderate activities included chlortetracycline with an MIC range of 0.25–16 μg/ml, lincomycin with an MIC range of 8–64 μg/ml, and tylosin with an MIC range of 0.5–2 μg/ml. The extracellular activity results showed that valnemulin had the highest activity against *L. intracellularis*; all isolates had MICs of $\leq$0.25 μg/ml. The antimicrobials that had moderate activity were carbadox with an MIC range of 1–4 μg/ml, chlortetracycline with an MIC range of 16–64 μg/ml, tiamulin with an MIC range of 1–4 μg/ml, and tylosin with an MIC range of 2–16 μg/ml. The antimicrobial that showed the least activity against *L. intracellularis* was lincomycin with MICs of 32–>128 μg/ml.

4. Discussion

Although methodologies for determining antimicrobial sensitivity of intracellular organisms have been developed, the methods and interpretations of their results have not been standardized or uniformly accepted. In this study, the practicality of assessing the *in vitro* antimicrobial activity against *L. intracellularis* was demonstrated using a tissue culture system, which was modified from a previous study (McOrist et al., 1995b).

The MIC results in the earlier studies testing various antimicrobials against *L. intracellularis* strains used only three strains from the United Kingdom. This was due to the limited number of strains available and the difficulty of the laboratory techniques for maintaining and culturing *L. intracellularis* (McOrist and Gebhart, 1995; McOrist et al., 1995b). One European isolate (916/91 or NCTC 12657) that was tested in the previous study (McOrist et al., 1995b) was also retested for antimicrobial activity in this study. Unfortunately, individual MIC endpoints were not shown for individual isolates in the previous study. Therefore, a direct comparison of the MIC endpoints of the current study to the earlier study is difficult to perform. With the exception of tylosin and tiamulin (intracellular MIC), the MIC results of the tested antimicrobials from the earlier study (McOrist et al., 1995b) were always within the range of a two-fold dilution compared to our results. In those studies and this current study, the extracellular and intracellular MICs for *L. intracellularis* were determined in an effort to mimic *L. intracellularis* infections in which the bacteria would be exposed to antimicrobials before and after invasion into intestinal cells. The results of the intracellular and extracellular MICs obtained from two different batches of each *L. intracellularis* strain demonstrated that the assay was reproducible. The median MIC from the two replicates was always within a two-fold dilution, determined by assessing whether any duplicate was more than a two-fold dilution away from the log 2 mean MIC (Table 1).

Currently there are no antimicrobial MIC breakpoints for intracellular organisms using a tissue culture system; therefore, interpretations of the sensitivity data are complicated. For various reasons, these data should only be used as a guide to determine which antimicrobials could be effective in treating *L. intracellularis* infections *in vivo*. First, it is unknown how the *in vitro* assay compares to *in vivo L. intracellularis* infections. Second, it is unknown what concentration of each antimicrobial can be attained

at the site of *L. intracellularis* infection. This concentration is critical in determining whether a specific isolate is susceptible or resistant. Finally, it is unknown whether the antimicrobial would have the greatest effect on *L. intracellularis* while the bacteria are extracellular or intracellular. The data can be used, however, to predict the utility of the antibiotic. If there is no diversity in activity levels and yet the MIC is very low, such as the observed variation in carbadox intracellular MIC values, then this antibiotic might function very well. If there is a large range of MICs, such as was observed with chlortetracycline intracellular MIC levels, then some *L. intracellularis* isolates may have less sensitivity to that antibiotic.

According to our results, extracellular MICs were higher than the intracellular MICs for all antimicrobials, while the previous report showed that both of them were similar (McOrist et al., 1995b). One explanation for this difference may be the effect of contact time with the antimicrobials. The extracellular activity assay was designed to have less contact time than the intracellular activity assay (i.e., one day compared to three consecutive days). These differing contact times were performed due to the fact that *L. intracellularis* can penetrate the cells within 24 h (Lawson et al., 1993; McOrist et al., 1995a). By definition, an extracellular MIC is the effect of the antimicrobial on *L. intracellularis* before the bacteria enter the cells and, therefore, only one day of exposure to the antimicrobial can be used to determine the extracellular MIC. A fraction of the *L. intracellularis* could have survived the single extracellular treatment and subsequently returned to active phase upon the disappearance of the antimicrobials. This could suggest that a single-dose antimicrobial treatment is insufficient to inhibit the growth of *L. intracellularis*. A potential explanation for the lower intracellular MIC is the accumulation of antimicrobial inside the cells, making the intracellular concentration much higher than the extracellular concentration. The increased intracellular concentrations could enhance the chemotherapeutic activity of antimicrobials against intracellular bacteria. In contrast to the macrolides, β-lactams, aminoglycosides and lincomycin are poorly accumulated intracellularly (Carryn et al., 2002, 2003). Although this study did not determine the antimicrobial concentration inside the cells, the intracellular MIC results seemed to show that most of the antimicrobials tested can penetrate and bind to internalized *L. intracellularis* to exert their chemotherapeutic action.

Interestingly, one observation from this study was that the North American *L. intracellularis* isolates tended to have higher intracellular and extracellular MICs than the European *L. intracellularis* isolates. Additional *L. intracellularis* isolates from North America and Europe will need to be tested before drawing any conclusions about geographic differences and antimicrobial sensitivities changing over time. This study evaluated most of the *L. intracellularis* isolates currently available globally and obtained from 2 different continents, including new clinical (low passage) and well-established (high passage) *L. intracellularis* isolates. This was important because intracellular bacteria maintained under a tissue culture system for an extended period of time have the potential to develop genomic mutations that could impact their antimicrobial sensitivities (Drancourt and Raoult, 1993). Therefore, these results represent the most comprehensive *L. intracellularis* MIC study to date. Further *in vivo* studies should be conducted to confirm the antimicrobial efficacies.

5. Conclusion

Our *in vitro* data greatly expand the antimicrobial MIC information available for *L. intracellularis*. Based on our *in vitro* results, it is clear that *L. intracellularis* isolates have a diversity of antimicrobial sensitivity patterns. Because it is unlikely that *L. intracellularis* will be isolated and tested during a PE outbreak, our data can serve as an *in vitro* guideline for the range of antimicrobial responses of *L. intracellularis*. Based on this guideline, we predict carbadox, tiamulin and valnemulin to be the most active antimicrobials, chlortetracycline and tylosin to be intermediately active, and lincomycin to be the least active antimicrobial against *L. intracellularis*.

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