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Short Communication

Recent antimicrobial susceptibility of *Lawsonia intracellularis* field isolates from pigs with proliferative hemorrhagic enteropathy in Korea

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Abstract

The study was performed to investigate the antimicrobial susceptibility of *L. intracellularis* field isolates from Korean pig farms. The novel four field isolates successfully obtained from clinical cases were prepared in IEC-18 cells to conduct the *in vitro* antimicrobial susceptibility testing (AST) by determining minimum inhibitory concentrations (MIC)s; either intracellular (InMIC) or extracellular MICs (ExMIC). The final MICs were assessed by counting the number of heavily infected cells (HICs;> 30 bacteria per cell) using an immunoperoxidase monolayer assay. Enrofloxacin (0.125 to 0.25 µg/mL: InMIC and 2 to 16 µg/mL; ExMIC) presented the most notable antimicrobial susceptibility, and marbofloxacin (0.25 to 0.5 µg/mL and 4 to 32 µg/mL) followed. Colistin (0.125 to 2 µg/mL and 2 to 4 µg/mL) presented a susceptibility followed by tylvalosin (0.5 to 1 µg/mL and 2 to 4 µg/mL). Florfenicol and lincomycin had the weakest susceptibility and amoxicillin, penicillin G, chlortetracycline, oxytetracycline, tiamulin, tilmicosin and tylosin displayed weak susceptibility. Dividing cells in culture and strict environmental conditions to isolate and cultivate *L. intracellularis* has limited the maintenance of this bacteria *in vitro* to only a few laboratories. Four isolates were successfully maintained in our laboratory, which enabled us to determine the antimicrobial susceptibility profile. In addition, the results could be one of the contributions in clinical fields.

Keywords: Antimicrobial susceptibility, *Lawsonia intracellularis,* Minimum inhibitory concentration, Pig, Porcine proliferative hemorrhagic enteropathy

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Introduction

Lawsonia (*L.*) *intracellularis* is a microaerophilic intestinal obligate intracellular bacterium causing proliferative hemorrhagic enteropathy (PHE) and proliferative intestinal adenomatosis (PIA) resulting in diarrhea, rough coat hair, anorexia, and growth retardation in finisher and grower pigs (Lawson and Gebhart, 2000). It has been detected in many other animals worldwide (Lawson and Gebhart, 2000; Park et al., 2015; Hossain et al., 2016; Oh et al., 2017). The subclinical form of PHE has not been easily recognized recently and it can develop as subacute or chronic at any moment under stressful condition. It is one of the most important diseases in the pig industry worldwide (Lawson and Gebhart, 2000).

Up to now, antimicrobial therapy remains the only treatment available. Tiamulin, tylosin, lincomycin, and chlortetracycline have been commonly recommended and used in the field (McOrist et al., 1995; Marsteller et al., 2001) and oxytetracycline, valnemulin, doxycycline, josamycin, and leucomycin have also been known to be effective (Tzika et al., 2009; Larsen et al., 2016) according to field experience not from exact *in vitro* antimicrobial susceptibility testing (AST). However, AST cannot be easily performed for *L. intracellularis* because it requires a complicated cell culture system and particular atmosphere for its growth and proliferation (McOrist et al., 1995; Yeh et al., 2011). In the last two decades, *in vitro* AST have been performed on ten field isolates from pigs in North America, three in the United Kingdom, and two in South Korea (McOrist et al., 1995; Wattanaphansak et al., 2009; Yeh et al., 2011).

In Korea, the antimicrobial susceptibility of *L. intracellularis* was tested first in 2006 (Yeh et al., 2006) and 2011 (Yeh et al., 2011). Since then, no further antimicrobial susceptibility patterns of *L. intracellularis* in Korea have been investigated presumably due to its being difficult to isolate and maintain *in vitro*. Therefore, the aim of this study is to update *in vitro* antimicrobial sensitivities of newly isolated *L. intracellularis* in Korea from 2013 to 2017 using 13 antimicrobial agents.

Materials and Methods

Bacterial strains and preparation: The four novel *L. intracellularis* field isolates were obtained from the hemorrhagic region of the small intestine from finisher pigs with PHE (CBNU001, CBNU002, and CBNU006) and lactating piglets (CBNU004) in 2013 to 2017. The isolates were prepared in IEC-18 cells (CRL 1589, ATCC, VA, USA) and harvested as previously described elsewhere (Lawson et al., 1993).

Antimicrobial sensitivity testing (AST) and data analysis: The AST was conducted by determining the minimum inhibitory concentrations (MIC)s of each antimicrobial against *L. intracellularis*. The MIC tests and results were studied according to previous studies with modifications in Korea (McOrist et al., 1995; Wattanaphansak et al., 2009; Yeh et al., 2011). In brief, antimicrobial agents used for the MICs were amoxicillin, penicllin G, chlortetracycline, oxytetracycline, colistin, enrofloxacin, marbofloxacin, florfenicol, lincomycin, tiamulin, tylosin (Sigma-Aldrich, MO, USA) and tylvalosin (Santa Cruz Biotechology, TX, USA), and all agents were serially diluted from 0.125 to 256 µg/mL. Briefly, to determine the intracellular MIC (InMIC), 100 µL of bacterial suspension was inoculated and incubated for 24 h in a 96-well plate which the IEC-18 cells were cultured. Antimicrobial stock solutions were added at 1, 2 and 3 day post inoculation (dpi) when the medium was freshly replaced. For the extracellular MIC (ExMIC), after exposure to each concentration of antimicrobials for 2 h, bacterial cells were infected to IEC-18 cells and cultured for 24 h. Then, the medium was replaced in new DMEM supplemented with L-glutamine and FBS (7%, v/v) and each antimicrobial agent to be tested at 1, 2, and 3 dpi. After that, the 96-well plates were fixed with cold acetone/methanol (1:1 v/v) and counted the number of heavily infected cells (HICs;> 30 bacteria per cell) using an immunoperoxidase monolayer assay staining method of Korea (McOrist et al., 1995; Wattanaphansak et al., 2009; Yeh et al., 20011).

Results and Discussion

InMIC and ExMIC: The results were determined by taking the median value from a set of triplicate 96-well plates and performing in duplicate. The InMIC and ExMIC values of each of the antimicrobials are displayed in Table 1. In the present study, the InMICs of the three *L. intracellularis* isolates were lower than ExMICs, which is consistent with previous studies in Korea (McOrist et al., 1995; Wattanaphansak et al., 2009). In all isolates, enrofloxacin displayed the greatest activity with InMICs ranging from 0.125 to 0.25 µg/mL of which its ExMICs were ranging from 2 to 16 µg/mL, followed by marbofloxacin with MICs of 0.25 to 0.5 µg/mL and 4 to 32 µg/mL. Also, colistin presented high susceptibilities with both of InMICs and ExMICs, 0.125 to 2 µg/mL and 2 to 4 µg/mL, respectively, followed by tylvalosin, MICs of 0.5 to 1 µg/mL and 2 to 4 µg/mL. Whereas, florfenicol and lincomycin had the weakest activity and amoxicillin, penicillin G, chlortetracycline, oxytetracycline, tiamulin, tilmicosin, and tylosin displayed weak activities with InMICs and ExMICs of 4 to 32 µg/mL and 16 to 64 µg/mL, respectively.

Changes in Antimicrobial sensitivity patterns: The result postulated that macrolides could be still an all right option of treatments for *L. intracellularis* in Korea. Previously, two field isolates of *L. intracellularis* in Korea presented higher intracellular and extracellular susceptibility to almost all antimicrobials including amoxicillin, penicillin G, chlortetracycline, lincomycin, tiamulin, tilmicosin, and tylosin Korea (Yeh et al., 20011). However, those macrolides and tylvalosin displayed a weak susceptibility to those isolates. Around that time six isolates from North America and four isolates from Europe displayed higher intracellular and extracellular susceptibilities to chlortetracycline, tylosin, and tiamulin (Wattanaphansak et al., 2009). Lincomycin presented very weak susceptibility to those five isolates from North America consistent with our results. In addition,

Table 1 Intracellular and extracellular MICs for 13 antimicrobial agents against four *L. intracellularis* isolates from pigs in Korea

Antimicrobial class	Antimicrobial agent(s)	MIC (μg/mL)[a]											
		CBNU001 (2013)		CBNU002 (2014)		CBNU004 (2016)		CBNU006 (2017)		PHE/KK421 (2002)[b]		PIA/MyCoyL1 (2010)[b]	
		InMIC[c]	ExMIC[d]	InMIC	ExMIC	InMIC	ExMIC	InMIC	ExMIC	InMIC	ExMIC	InMIC	ExMIC
Penicillins													
	Amoxicillin	8	32	8	32	8	32	16	32	0.5	8	2~4	16
	Penicillin G	32	64	16	64	16	64	16	64	1~2	2~4	4	16
Tetracyclines													
	Chlortetracycline	16	64	32	64	16	64	32	64	2~4	16	8	64
	Oxytetracycline	16	64	16	64	16	64	8	64				
Polypeptides													
	Colistin	2	2	0.125	4	0.125	2	0.5	2				
Fluroquinolones													
	Enrofloxacin	0.25	16	0.125	2	0.125	4	0.25	4	2	8	2~4	16
	Marbofloxacin	0.5	32	0.5	4	0.25	4	0.5	8				
Phenicols													
	Florfenicol	>256	>256	>256	>256	64	>256	128	>256				
Lincosamide													
	Lincomycin	>256	>256	128	>256	64	128	128	>256	16	64	>128	>128
Pleuromutilins													
	Tiamulin	8	16	16	64	32	64	8	32	0.25~0.5	4~8	2	32
Macrolides													
	Tilmicosin	4	32	4	32	16	64	16	32	0.125	0.5	0.125	0.25~0.5
	Tylosin	16	64	8	64	32	64	16	32	0.25~0.5	1	0.25	1
	Tylvalosin	1	2	0.5	2	1	4	1	4				

[a]The MIC data of each antimicrobial were determined using the median value from a set of triplicate 96-well plates and performed in duplicate on each *L. intracellularis* isolates independently.
[b]Yeh et. al., 2011; [c]The intracellular MIC; [d]The extracellular MIC.

tylvalosin oral treatment was studied to be effective to control PHE associated with *L. intracellularis* in pig farms (Canning et al., 2016), consistent with our *in vitro* MIC results. Tylvalosin was registered in Korea for other pathogenic bacteria, not for *L. intracellularis* control but an experimental MIC test was conducted to compare with other studies. Larsen et al. (2016) reported that the occurrence of diarrhea and fecal shedding was reduced after *in vivo* oxytetracycline treatment in nursery pigs infected with *L. intracellularis* but this is contrary to our results displaying the weakest susceptibility to oxytetracycline. Fluroquinolones showed the greatest susceptibility and were used to control PHE outbreaks in the very farm where the isolates came from. However, the case was not fully controlled with the antimicrobial. Based on the opinions of various microbiologists, it is cautiously considered that frequently used antimicrobial agents can exert selective pressure, allowing the bacteria with inherent resistance or newly acquired mutations or resistance genes to survive and proliferate (Aminov, 2009). Colistin showed a strong susceptibility to the *L. intracellularis* isolates, being the first MIC report of colistin to the best of the authors' knowledge. However, it is considered that the use of colistin should be cautious to prevent the transmission of multidrug resistance genes to other bacteria in the same or different animals, to the food chain, and to the human community (Lim et al., 2016).

For each class of antimicrobial agents, all the four isolates had moderate to high resistance to penicillins, tetracyclines, polypeptides, fluroquinolones, phenicols, lincosamides, pleuromutilins, and macrolides. Due to the difficulty of culturing and maintaining *L. intracellularis*, studies on antimicrobial susceptibility against *L. intracellularis* are still limited in a few laboratories. That is why there have been only few studies on the bacterial antimicrobial resistance patterns worldwide (McOrist et al., 1995; Wattanaphansak et al., 2009; Yeh et al., 2011). Until now, recommended antimicrobials for the treatment of PHE with *L. intracellularis* in herds have not been based on antimicrobial susceptibility testing *in vitro*, but on assumptions from presumptive a few clinical trials with commonly used antimicrobials in field. It is noteworthy to see the resistance patterns of macrolides and lincosamides have long been treatment options against *L. intracellularis* infection in Korean pig farms. High resistance to antimicrobials of recent *L. intracellularis* isolates in Korea leads to the conclusion that antibiotics should be prescribed properly and cautiously.

Based on changes in antibiotic resistance or susceptibility patterns of commonly used antibiotics in pig farms and the increased focus on reducing antibiotic use, it is concluded that precise use of antibiotics is important. In addition, a variety of other preventive measures, including the use of vaccines, good hygiene, proper nutrition, and environmental improvement, should be accompanied to protect pigs from the pathogen. The four isolates of this study could not be said to represent all of field *Lawsonia* spp., but more isolates will continue to be conducted to update the results of antibiotic resistance.

Competing interests: None declared.

Ethical approval: Not required.

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