NOTE *Bacteriology*

Application of Enrofloxacin and Orbifloxacin Disks Approved in Japan for Susceptibility Testing of Representative Veterinary Respiratory Pathogens

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ABSTRACT. In this study, susceptibilities of *Pasteurella multocida*, *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae* to enrofloxacin and orbifloxacin were tested using an agar diffusion method with the commercial disks and a broth microdilution method. Good correlation between the 2 methods for enrofloxacin and orbifloxacin was observed for *P. multocida* (r = −0.743 and −0.818, respectively), *M. haemolytica* (r = −0.739 and −0.800, respectively) and *A. pleuropneumoniae* (r = −0.785 and −0.809, respectively). Based on the Clinical and Laboratory Standards Institute interpretive criteria for enrofloxacin, high-level categorical agreement between the 2 methods was found for *P. multocida* (97.9%), *M. haemolytica* (93.8%) and *A. pleuropneumoniae* (92.0%). Our findings indicate that the tested commercial disks can be applied for susceptibility testing of veterinary respiratory pathogens.

KEY WORDS: *Actinobacillus pleuropneumoniae*, agar disk diffusion, antimicrobial susceptibility testing, *Mannheimia haemolytica*, *Pasteurella multocida*

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Enrofloxacin and orbifloxacin are veterinary fluoroquinolone drugs used to treat cattle and pigs in Japan. The drugs have been approved as antimicrobials that possess efficacy against several bacterial infectious diseases, including bovine bacterial pneumonia due to *Pasteurella multocida* and *Mannheimia haemolytica* and porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* [10].

The World Health Organization proposed that fluoroquinolone drugs should be classified as critically important antimicrobials for both humans and animals [4]. In addition, the Food Safety Commission categorized the drugs as class I antimicrobials (i.e., critically important antimicrobials) [5]. For these reasons, veterinarians are strongly encouraged to prudently use veterinary fluoroquinolones as second-line drugs and apply the drugs only to bacterial pathogens categorized as susceptible based on the results of susceptibility testing. To facilitate the prudent use of veterinary fluoroquinolones, disks of enrofloxacin and orbifloxacin for susceptibility testing (i.e., VKB Disk Eiken Enrofloxacin® and VKB Disk Eiken Orbifloxacin®, respectively, Eiken Chemical Co., Ltd., Tochigi, Japan) were recently approved in Japan. However, the applicability of these commercial disks for susceptibility testing of veterinary respiratory pathogens remains to be validated.

In this study, we tested enrofloxacin and orbifloxacin susceptibilities of bovine and swine strains of *P. multocida*, *M. haemolytica* and *A. pleuropneumoniae* by an agar diffusion method using commercially available disks and compared the results with those of a broth microdilution method.

A total of 146 clinical strains consisting of 48 bovine strains of *P. multocida*, 48 strains of *M. haemolytica* and 50 porcine strains of *A. pleuropneumoniae* were included in this study. In addition, *Escherichia coli* ATCC 25922 and *A. pleuropneumoniae* ATCC 27090 were used as quality control strains for susceptibility testing.

Using 5-μg enrofloxacin (VKB Disk Eiken Enrofloxacin®) and 10-μg orbifloxacin disks (VKB Disk Eiken Orbifloxacin®), agar disk diffusion susceptibility testing was performed for each strain following the Clinical and Laboratory Standards Institute (CLSI) guidelines [2]. Each strain was directly suspended in sterile saline at a concentration of McFarland 0.5. For *P. multocida* and *M. haemolytica*, the bacterial suspension was spread with a cotton swab over Mueller–Hinton agar with 5% sheep blood (Eiken Chemical Co., Ltd.). After placing the disks for both antibiotics, the plates were incubated at 35°C in ambient air for 20–24 hr. For *A. pleuropneumoniae*, a suspension of each strain was spread with a cotton swab over chocolate agar, which was prepared by heating after 5% horse blood had been added to the Mueller–Hinton agar (Becton, Dickinson and Co., Ltd., Sparks, MD, U.S.A.). After placing the disks for both antibiotics, the plates were incubated at 35°C in 5% CO_2 for 20–24 hr.

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Table 1. Minimum Inhibitory Concentration (MIC) Distributions of Enrofloxacin and Orbifloxacin for the Tested Organisms

Organism (No. of strains)	MIC range (μg/m*l*)	Number of strains with MIC (μg/m*l*) of:												MIC_{50}	MIC_{90}
		≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32		
Enrofloxacin																
Pasteurella multocida (48)	≤0.015–4	37	0	3	5	1	0	0	1	1	0	0	0	0	≤0.015	0.12
Mannheimia haemolytica (48)	0.03–16	0	25	4	1	4	11	0	0	0	0	3	0	0	0.03	0.5
Actinobacillus pleuropneumoniae (50)	≤0.015–16	1	32	1	3	6	2	0	0	4	0	1	0	0	0.03	0.5
Orbifloxacin																
Pasteurella multocida (48)	≤0.015–16	31	5	2	2	6	0	0	0	1	0	1	0	0	≤0.015	0.25
Mannheimia haemolytica (48)	≤0.015–16	2	27	0	1	4	6	5	0	0	0	3	0	0	0.03	1
Actinobacillus pleuropneumoniae (50)	≤0.015–16	1	28	5	0	2	8	1	0	1	3	1	0	0	0.03	1

Broth microdilution testing was performed using a custom-designed frozen plate, which was prepared by Eiken Chemical Co., Ltd. according to the CLSI guidelines [2]. In the frozen plates, enrofloxacin (Sigma-Aldrich Co. LLC., St. Louis, MO, U.S.A.) and orbifloxacin (Hayashi Pure Chemical Ind., Ltd., Osaka, Japan) were prepared in serial 2-fold dilutions ranging from 0.015 to 32 μg/m*l* in cation-adjusted Mueller–Hinton broth (CAMHB). The final test concentration of bacteria was approximately 5×10^5 colony-forming units/m*l*, and minimum inhibitory concentrations (MICs) were read after 20–24 hr incubation at 35°C. For testing *A. pleuropneumoniae* strains, CAMHB was supplemented with 2% lysed horse blood, 5 mg/m*l* yeast extract and 15 μg/m*l* nicotinamide adenine dinucleotide by Eiken Chemical Co., Ltd.

The result of susceptibility testing for enrofloxacin was interpreted using the CLSI guidelines [3] as follows: the zone diameters and MIC breakpoints were ≥21 mm and ≤0.25 μg/m*l* for susceptible, 17–20 mm and 0.5–1 μg/m*l* for intermediate and ≤16 mm and ≥2 μg/m*l* for resistant for *P. multocida* and *M. haemolytica* and ≥23 mm and ≤0.25 μg/m*l* for susceptible, 19–22 mm and 0.5 μg/m*l* for intermediate and ≤18 mm and ≥1 μg/m*l* for resistant for *A. pleuropneumoniae*.

Data were displayed as scattergrams with zone diameters on the x-axis and MICs on the y-axis illustrating each species. Correlations between the zone diameters and MICs were confirmed by regression analysis. Regression lines were calculated excluding off-scale MICs (>32 and ≤0.03 μg/m*l*) and zone diameters of ≤6 mm according to a previous study [6].

In Japan, enrofloxacin resistance has been relatively less prevalent in veterinary respiratory pathogens including *P. multocida* [7], *M. haemolytica* [8] and *A. pleuropneumoniae* [9]. Most of the tested strains in this study were also highly susceptible to enrofloxacin based on the low MIC_{50} and MIC_{90} values for this drug (Table 1). On the contrary, susceptibility to orbifloxacin has not yet been investigated in veterinary respiratory pathogens. Our results illustrated that the MIC_{50} and MIC_{90} values of orbifloxacin were very similar to those of enrofloxacin. These data indicate that orbifloxacin has similar *in vitro* activity as enrofloxacin against these pathogens.

Scattergrams of the MICs and corresponding zone diameters for enrofloxacin and orbifloxacin were constructed (Figs. 1 and 2, respectively). Among the tested bacterial species, *P. multocida* had the largest zone diameters for both drugs (16–56 mm for enrofloxacin and 11–55 mm for orbifloxacin) relative to *M. haemolytica* (7–35 and 7–38 mm, respectively) and *A. pleuropneumoniae* (≤6–33 and 13–35 mm, respectively). Such differences in zone diameters among bacterial species agreed with the results of the microdilution method in which *P. multocida* had relatively lower MIC_{50} and MIC_{90} values for the 2 drugs than the other 2 pathogens. In addition, the correlation between zone diameters and MICs for enrofloxacin and orbifloxacin was calculated. The 3 pathogens had similar correlation coefficients for each drug. For all 3 bacterial pathogens, the correlation coefficients for enrofloxacin and orbifloxacin were statistically significant ($P<0.01$, Student's *t*-test). These data indicate that both the tested disks are applicable for evaluating susceptibility of *P. multocida*, *M. haemolytica* and *A. pleuropneumoniae* to each drug.

Based on CLSI interpretive criteria for enrofloxacin, all tested organisms were categorized as susceptible, intermediate or resistant by an agar diffusion method using the commercial disk and a microdilution method. As a result, extremely high categorical agreements (i.e., >90%) between the 2 methods were found for *P. multocida*, *M. haemolytica* and *A. pleuropneumoniae* (Table 2). Thus, the tested enrofloxacin disk is likely to provide correct categorization (i.e., susceptible, intermediate and resistant) for the 3 pathogens, if the disk is tested using the agar disk diffusion method according to the CLSI guidelines. On the contrary, the interpretive criteria for orbifloxacin for veterinary respiratory pathogens have not been internationally established, because this drug has been approved for livestock only in Japan [10]. The development of animal species-bacterial pathogen breakpoints is based on 3 components: 1) the therapeutic outcome of treatment, 2) pharmacokinetics (PK)-pharmacodynamics (PD) and 3) *in vitro* susceptibility testing [1, 11]. In addition to the *in vitro* susceptibility results for orbifloxacin in this study, further studies including clinical investigation and PK-PD analysis are needed to establish the breakpoint of this drug for veterinary respiratory pathogens.

In conclusion, we evaluated the usefulness of the commercially available disks of enrofloxacin and orbifloxacin for *P. multocida* and *M. haemolytica* of bovine origin and

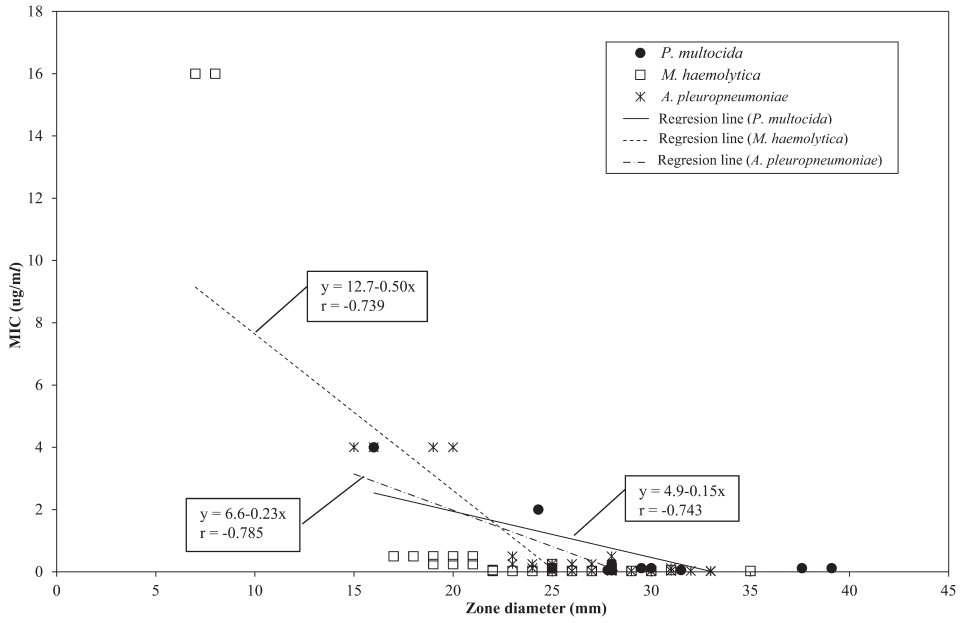

Fig. 1. Enrofloxacin scattergram of the zone diameters of 5-μg enrofloxacin disks versus the minimum inhibitory concentrations (MICs) for *P. multocida*, *M. haemolytica* and *A. pleuropneumoniae* strains.

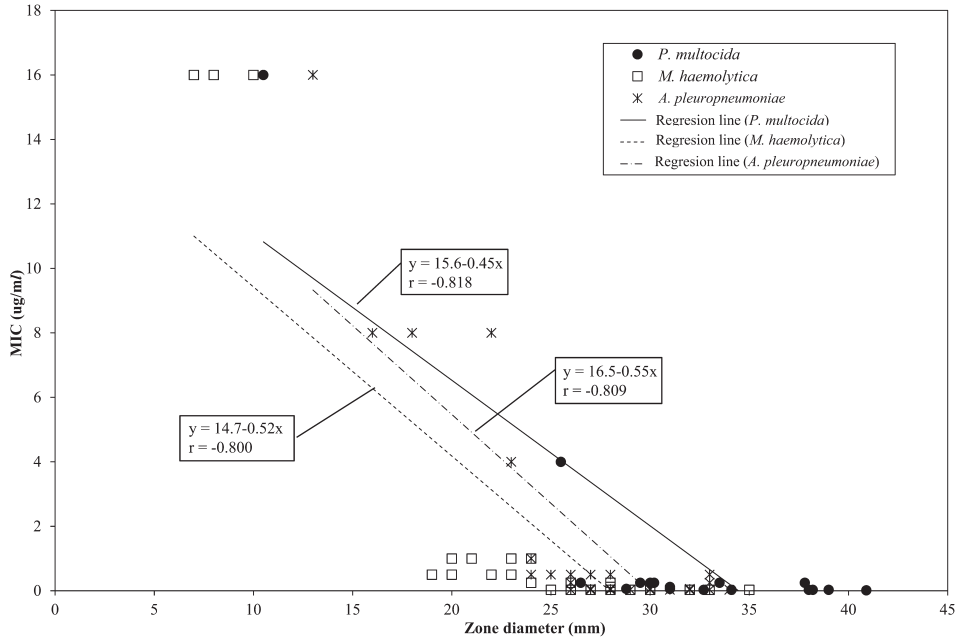

Fig. 2. Orbifloxacin scattergram of the zone diameters of 10-μg orbifloxacin disks versus the minimum inhibitory concentrations (MICs) for *P. multocida*, *M. haemolytica* and *A. pleuropneumoniae* strains.

A. pleuropneumoniae of swine origin. The agar diffusion method results of these disks were correlated with those of the broth microdilution method for these respiratory pathogens. Therefore, our data provide strong evidence that susceptibility tests using the commercial disks can be applied to the selection of appropriate antimicrobials, resulting in evidence-based veterinary medicine.

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Table 2. Categorical Agreement between the Agar Disk Diffusion and Broth Microdilution Results for Enrofloxacin

Organism (No. of strains)	No. of strains (%)						Categorical agreement (%)
	Broth microdilution			Agar disk diffusion			
	S	I	R	S	I	R	
Pasteurella multocida (48)	46 (95.8)	0 (0)	2 (4.2)	47 (97.9)	0 (0)	1 (2.1)	97.9
Mannheimia haemolytica (48)	34 (70.8)	11 (22.9)	3 (6.3)	33 (68.8)	12 (25.0)	3 (6.3)	93.8
Actinobacillus pleuropneumoniae (50)	43 (86.0)	2 (4.0)	5 (10.0)	45 (90.0)	2 (4.0)	3 (6.0)	92.0

S, susceptible; I, intermediate; R, resistant.

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