NOTE *Bacteriology*

Recent Trends in Antimicrobial Susceptibility and the Presence of the Tetracycline Resistance Gene in *Actinobacillus pleuropneumoniae* Isolates in Japan

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ABSTRACT. A total of 101 *Actinobacillus pleuropneumoniae* isolates from diseased pigs taken from across Japan during 2002 to 2005 were examined for antimicrobial susceptibility. All isolates were susceptible to ceftiofur, erythromycin, florfenicol and enrofloxacin. Antimicrobial-resistant isolates to oxytetracycline (OTC) (27.7%), dihydrostreptomycin (10.9%), thiamphenicol (10.9%), kanamycin (5.9%), trimethoprim (4.0%) and ampicillin (2.0%) were recognized. OTC-resistant isolates taken from 1986 to 2005 were examined for the tetracycline resistance gene. In OTC-resistant isolates, *tetB* has been the most frequently isolated gene in Japan. It is likely that the dissemination of *tetB* has contributed to the increased OTC resistance of *A. pleuropneumoniae* in Japan.

KEY WORDS: *Actinobacillus pleuropneumoniae*, antimicrobial susceptibility, *tet* gene.

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A. pleuropneumoniae is the causative agent of porcine pleuropneumonia that causes great economic losses in the pig industry worldwide [9]. In Japan, several inactivated vaccines are available for prevention of the disease. However, even after the approved commercialization of the vaccines, antimicrobial treatment has remained essential for the actual control of porcine pleuropneumonia with clinical signs under field conditions. Antimicrobial resistance in *A. pleuropneumoniae* has been found worldwide [3–5, 7, 14]. In Japan, antimicrobial-resistant phenotypes of *A. pleuropneumoniae* tend to be characteristic in each serovar. Isolates of serovars 1 and 5 are frequently resistant to tetracycline, although most serovar 2 isolates are susceptible to antimicrobials [7, 14]. As the frequency of antimicrobial resistance in *A. pleuropneumoniae* increases [7], it will be difficult to select antimicrobial agents for treatment and control of the disease.

Tetracycline antibiotics were approved in the 1960's and are still the most frequently used ones for treatment of porcine diseases in Japan [12]. Tetracycline resistance in *A. pleuropneumoniae* has been frequently found since the 1990's [7, 14]. Tetracycline resistance is generally associated with the acquisition of tetracycline resistance genes (*tet* genes) in bacteria [6]. In *A. pleuropneumoniae*, *tetB* has been frequently detected in several countries [2, 13], although a variety of *tet* genes has been found among several serovar isolates [2]. However, there is little available information on the type of *tet* genes in tetracycline-resistant *A. pleuropneumoniae* in Japan. The purpose of this study was to determine recent nationwide trends in serovars, antimicrobial susceptibility, and the characteristics of the *tet* genes of *A. pleuropneumoniae* in Japan.

A total of 101 isolates were obtained from pleuropneumonic lesions of pigs submitted for routine diagnosis at Livestock Hygiene Service Centers in 26 Japanese prefectures from 2002 to 2005. Serotyping against serovars 1 to 12 and 15 was conducted by agar gel diffusion testing as previously described [1, 11]. Of the 101 strains, serovar 2 (66 isolates from 24 prefectures) was the most common, followed by serovar 5 (14 isolates from 8 prefectures), serovar 1 (8 isolates from 6 prefectures) and serovar 15 (2 isolates from 2 prefectures). The remaining eleven isolates were untypable. We also examined for serovars of 125 isolates from pleuropneumonic lesions of pigs from 1999 to 2000 [7]. From 1999 to 2000, 24 isolates of serovar 1 came from 9 prefectures including 2 prefectures where serovar 1 was isolated from 2002 to 2005. The proportion of serovar 1 had significantly decreased from 19.2% during 1999 to 2000 [7] to 7.9% during 2002 to 2005 (χ^2 test, $p<0.05$).

Minimum inhibitory concentrations (MICs) were determined by an agar dilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) [8]. *A. pleuropneumoniae* ATCC 27090 was used as a control strain. The breakpoints used for ceftiofur (CTF) and kanamycin (KM) were adopted from the CLSI recommendations. For the remaining antimicrobials, the midpoint of MIC peaks was defined in this study as the resistant breakpoint when the MIC distribution of antimicrobials was bimodal.

Resistant strains were recognized for oxytetracycline (OTC) (27.7%), dihydrostreptomycin (DSM) (10.9%), thiamphenicol (TP) (10.9%), KM (5.9%), trimethoprim (TMP) (4.0%) and ampicillin (ABPC) (2.0%) (Table 1). All

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Table 1. Antimicrobial susceptibility of 101 *A. pleuropneumoniae* isolates

Antimicrobial agents[b)]	Minimum Inhibitory Concentration (μg/m*l*)			MIC breakpoint of resistance	No. of resistant strains (%)	MIC of ATCC 27090 (n=6)[a)]
	Range	50%	90%			
ABPC	≤0.125–128	≤0.125	0.25	2	2 (2.0)	≤0.125
CTF	≤0.125	≤0.125	≤0.125	8		≤0.125
DSM	1–>512	8	128	64	11 (10.9)	8
KM	1–>512	8	16	64	6 (5.9)	8
EM	0.25–8	4	8			8
OTC	0.25–256	1	32	8	28 (27.7)	1
TMP	≤0.125–128	0.5	2	8	4 (4.0)	0.25–0.5
TP	0.5–512	0.5	32	8	11 (10.9)	1
FF	0.25–2	0.5	1			0.5–1
ERFX	≤0.125–0.25	≤0.125	≤0.125			≤0.125

a) Number of tested times.
b) ABPC: ampicillin, CTF: ceftiofur, DSM: dihydrostreptomycin, KM: kanamycin, EM: erythromycin, OTC: oxytetracycline, TMP: trimethoprim, TP: thiamphenicol, FF: florfenicol, ERFX: enrofloxacin.

Table 2. Antimicrobial resistance patterns and serovar of 101 *A. pleuropneumoniae* isolates

Number of resistant agents	Antimicrobial resistance patterns	Serovar					Total (%)
		1	2	5	15	UT[a)]	
0	Susceptible (%)	2[b)] (25.0)	52 (78.8)	2 (14.3)	0 (0.0)	8 (72.7)	64 (63.4)
1–4	Resistant (%)	6 (75.0)	14 (21.2)	12 (85.7)	2 (100.0)	3 (27.3)	37 (36.6)
1	DSM		2				2 (2.0)
	OTC		6	10			16 (15.8)
	TMP		1			1	2 (2.0)
	TP		3				3 (3.0)
2	ABPC+TP	1					1 (1.0)
	DSM+KM		1				1 (1.0)
	OTC+DSM		1				1 (1.0)
	OTC+TP	2					2 (2.0)
	OTC+TMP			2			2 (2.0)
3	OTC+DSM+TP	1				1	2 (2.0)
	OTC+DSM+KM				1		1 (1.0)
4	OTC+DSM+KM+ABPC				1		1 (1.0)
	OTC+DSM+KM+TP	2				1	3 (3.0)

a) Untypable.
b) Number of isolates.

isolates were susceptible to CTF, erythromycin (EM), florfenicol (FF) and enrofloxacin (ERFX). The frequency of OTC-resistant isolates was the highest among the antimicrobial agents tested. In this study, the frequencies of resistance to penicillin antibiotics in β-lactam antibiotics (resistant rate in 1999–2000: 12.0–13.6%→ in 2002–2005: 2.0%), OTC (44.8%→27.7%) and TP (30.4%→10.9%) were significantly lower than those from 1999 to 2000 [7] (χ^2 test, $p<0.01$). Since resistance frequencies to penicillin antibiotics, OTC and TP were higher in isolates of serovar 1 than those of serovar 2 (Table 2), as in previous studies [7, 14], the reduced number of isolates of serovar 1 was responsible in part for the change in resistance frequencies of the antimicrobials in the isolates studied. In our investigation regarding serovar, the frequencies of antimicrobial resistance in serovars 1, 2 and 5 were similar to those previously reported [7]. Isolates of serovar 5 showed a high frequency of resistance to OTC, as previously reported [7].

Eighty OTC-resistant isolates were examined for *tet* genes. Ten of the 80 OTC-resistant isolates were collected from lungs lesions of pigs slaughtered from 1986 to 1987 [10]. Forty-two and 28 OTC-resistant isolates were collected from pigs with pleuropneumonia from 1999 to 2000 [7] and from 2002 to 2005, respectively. Polymerase chain reaction (PCR) was applied for the detection of *tetA, tetB, tetH, tetL, tetM, tetO* and *tetS*, as previously described [2]. Of the 80 OTC-resistant isolates, 61, 6, 5 and 1 carried *tetB, tetH, tetO* and *tetA*, respectively (Table 3). Seven isolates were negative for all the genes tested. The *tetB* gene was the

Table 3. Tetracycline resistant gene isolated in different years

tet gene	Serovar	Number of isolates during:			Total
		1986–1987	1999–2000	2002–2005	
A	2	0	0	1	1
B	1	0	11	5	61
	2	0	11	0	
	5	1	13	12	
	6	0	1	0	
	15	–[b)]	–	2	
	UT	0	3	2	
	Subtotal	1	39	21	
H	2	2	0	4	6
O	2	0	1	0	5
	7	2	2	0	
	Subtotal	2	3	0	
ND[a)]	2	5	0	2	7
Total		10	42	28	80

a) Not detectable.
b) Not tested.

one most frequently found in Japan, as it is in Spain [2]. The *tetB* gene was found in isolates of serovars 1, 2, 5, 6 and 15 accounting for 92.9% (36 / 42) and 75.0% (17 / 28) of the OTC-resistant isolates from 1999 to 2000 and 2002 to 2005, respectively. These results showed that *tetB* was the predominant one in the isolates in 1999. Since the 1990's, tetracycline resistance has been most frequently found in Japanese isolates of *A. pleuropneumoniae* [7]. Thus, the increase of tetracycline resistance in Japan may be associated with the dissemination of *tetB* among *A. pleuropneumoniae* isolates.

Isolates of different origins were used to examine whether the types of *tet* genes were different or not. For investigation into the origins of isolates, isolates of serovars 2 and 5 from lungs of slaughtered pigs carried *tetH* and *tetB*, respectively, as did the isolates from pigs with pleuropneumonia. During the period of our investigation, resistance genes such as *tetA, tetB, tetH* and *tetO* were found in isolates of serovar 2. All OTC-resistant isolates of serovars 1, 5, 6 and 15 carried *tetB*, while the OTC-resistant isolates of serovar 7 carried *tetO*. In Spain, serovar 1 was found to carry *tetL*, serovar 2 *tetB*, serovar 6 *tetO* and serovar 7 *tetB* and *tetO* [2]. Thus, the relationship between serovar and resistant gene appears to differ by country.

Interestingly, *tetB* was found in 11 of 12 OTC-resistant isolates of serovar 2 in those collected during 1999 to 2000, but 5 of 7 OTC-resistant isolates carried either *tetA* or *tetH* in the 2002 to 2005 samples. Isolates of serovar 2 from lesions of pigs with pleuropneumonia collected during 1999 to 2000 and 2002 to 2005 carried different *tet* genes. TP resistance in isolates of serovar 2 significantly decreased from 21.1% (16 / 76) in the 1999 to 2000 isolates [7] to 4.5% (3/66) in the 2002 to 2005 ones. Nine (69.2%) of the 13 OTC-resistant isolates of serovar 2 from the 1999 to 2000 samples exhibited TP resistance [7], but such an isolate of serovar 2 was not isolated from the 2002 to 2005 ones. The reason for the change of resistance in serovar 2 is unknown. In *A. pleuropneumoniae*, most *tet* genes exist on plasmids [2]. It will be essential to examine genotypes and plasmid profiles in isolates of serovar 2 to clarify the reasons for the change of resistance.

This is the first report from Japan of antimicrobial susceptibility testing of *A. pleuropneumoniae* isolates performed by the CLSI method. The resistant breakpoints and MICs ranges in this study had almost the same values as those of previous studies that used the Japanese Society of Chemotherapy method [7, 8]: therefore, antimicrobial susceptibility data from the two methods were comparable.

In conclusion, antimicrobial susceptibility in this study did not show changes when compared with previous results [7], except for the frequencies of resistance against the penicillin antibiotics, OTC and TP. Though *tetB* was the one most frequently found in Japan, similar to Norway and Spain [2, 13], the relationship between serovars and resistant genes differed by country. The present results also indicated that dissemination of *tetB* in *A. pleuropneumoniae* contributed to the increase of tetracycline resistance in Japan.

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REFERENCES

1. Blackall, P. J., Klaasen, H. L. B. M., Van Den Bosch, H., Kuhnert, P. and Frey, J. 2002. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. *Vet. Microbiol.* **84**: 47–52.

2. Blanco, M., Gutiérrez-Martin, C. B., Rodríguez-Ferri, E. F., Roberts, M. C. and Navas, J. 2006. Distribution of tetracycline resistance genes in *Actinobacillus pleuropneumoniae* isolates from Spain. *Antimicrob. Agents Chemother.* **50**: 702–708.
3. Chang, C. F., Yeh, T. M., Chou, C. C., Chang, Y. F. and Chiang, T. S. 2002. Antimicrobial susceptibility and plasmid analysis of *Actinobacillus pleuropneumoniae* isolated in Taiwan. *Vet. Microbiol.* **84**: 169–177.
4. Gutiérrez-Martin, C. B., del Blanco, N. G., Blanco, M., Navas, J. and Rodríguez-Ferri, E. F. 2006. Changes in antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Spain during the last decade. *Vet. Microbiol.* **115**: 218–222.
5. Kim, B., Min, K., Choi, C., Cho, W. S., Cheon, D. S., Kwon, D., Kim, J. and Chae, C. 2001. Antimicrobiol susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Korea using new standardized procedures. *J. Vet. Med. Sci.* **63**: 341–342.
6. Levy, S. B., McMurry, L. M., Barbosa, T. M., Burdett, V., Courvalin, P., Hillen, W., Roberts, M. C., Rood, J. I. and Taylor, D. E. 1999. Nomenclature for new tetracycline resistance determinants. *Antimicrob. Agents Chemother.* **43**: 1523–1524.
7. Morioka, A., Asai, T. and Takahashi, T. 2006. Antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolates during 1999 and 2000 in Japan. *J. Jpn. Vet. Med. Assoc.* **59**: 815–819.
8. National Committee for Clinical Laboratory Standards. 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard –Second Edition (M31-A2).
9. Sebunya, T. N. K. and Saunders, J. R. 1983. *Haemophilus pleuropneumoniae* infection in swine: A review. *J. Am. Vet. Med. Assoc.* **182**: 1331–1337.
10. Suzuki, S., Ohmae, K., Ohishi, K., Muramatsu, M. and Takahashi, T. 1989. Antimicrobial susceptibility of *Actinobacillus (Haemophilus) pleuropneumoniae* isolated from pigs with pleuropneumonia. *Jpn. J. Vet. Sci.* **51**: 450–452.
11. Suzuki, S., Takahashi, T., Muramatsu, M., Ohishi, K., Nakajima, M. and Yamashita, M. 1988. Serotype of *Actinobacillus (Haemophilus) pleuropneumoniae* isolates from pigs in slaughter-house. *Jpn. J. Vet. Sci.* **50**: 1264–1267.
12. Tamura, Y. 2003. Trends in antimicrobial agents for veterinary use and control measures of antimicrobial resistance. *J. Jpn. Vet. Med. Assoc.* **56**: 685–691.
13. Wasteson, Y., Roe, D. E., Falk, K. and Roberts, M. C. 1996. Characterization of tetracycline and erythromycin resistance in *Actinobacillus pleuropneumoniae*. *Vet. Microbiol.* **48**: 41–50.
14. Yoshimura, H., Takagi, M., Ishimura, M. and Endoh, Y. S. 2002. Comparative *in vitro* activity of 16 antimicrobial agents against *Actinobacillus pleuropneumoniae*. *Vet. Res. Commun.* **26**: 11–19.