Antimicrobial Susceptibility of Pathogenic *Escherichia coli* Isolated from Sick Cattle and Pigs in Japan

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ABSTRACT. We examined the 12 antimicrobial susceptibilities of 175 *E. coli* isolates from sick cattle and pigs by an agar dilution method. Resistance was found in 78.3% of isolates for oxytetracycline, 70.3% of isolates for dihydrostreptomycin, and 49.1% of isolates for ampicillin. When compared with healthy animals reported by Kijima-Tanaka *et al.*, resistance rates for 11 antimicrobial agents were higher in sick cattle than in healthy cattle, and resistance rates for all antimicrobial agents were higher in sick pigs than in healthy pigs. Comparing cattle and pigs, resistance rates to colistin was higher in porcine isolates than in bovine isolates, but was lower in porcine isolates than in bovine isolates for cefazolin. With regard to the association of virulence factors, higher resistance rates to colistin and enrofloxacin were observed in STEC (61 strains) than in non-STEC (57 strains) among porcine isolates, while there were no significant differences in bovine isolates. In conclusion, these results can be considered helpful for adequate selection and prudent use of antimicrobial agents for several types of colibacillosis.
KEY WORDS: antimicrobial resistance, *Escherichia coli*, non-STEC, STEC.

Colibacillosis, caused by pathogenic *Escherichia coli*, is one of the significant diseases in the cattle and pig industries that leads to various clinical symptoms based on age, species of animal, and type of pathogenic factors, such as toxins. Diarrhea is the most common symptom of colibacillosis in livestock. Colibacillary diarrhea in pigs is classified into two types: neonatal diarrhea, which occurs in pigs two-weeks-old or younger, and post-weaning diarrhea, which occurs in pigs within two weeks after weaning [12]. Edema disease, also known as systemic edema, manifests as nervous symptoms or sudden death within 1–2 weeks after weaning as a result of infection with enterotoxemic *E. coli* (ETTEC), which is classified as shiga-toxin producing *E. coli* (STEC) [1, 12, 20].

As the spread of antimicrobial resistance among food-producing animals is a great concern for human and animal hygiene [2, 5, 16–18], the Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) program was established in 1999 [19], and it evaluates antimicrobial susceptibility of animal originating *E. coli* by an agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) [14]. Animals infected with pathogenic *E. coli* sometimes show clinical symptoms, becoming an antimicrobial therapeutic target. Therefore, this test was carried out for the purpose of evaluating antimicrobial susceptibility in pathogenic *E. coli* isolates. We determined the minimum inhibitory concentration (MIC) of *E. coli* isolates from sick animals using these guidelines, and the results were compared with those of healthy animals and evaluated for the presence of virulence factors. As a result, we found characteristic antimicrobial susceptibility for pathogenic *E. coli*.

MATERIALS AND METHODS

Bacterial isolates: A total of 175 *E. coli* isolates from individual cattle (57 isolates) and pigs (118 isolates) were collected by the Livestock Hygiene Service Centers of each prefecture across Japan from April 2001 to March 2004. *E. coli* was isolated from the lesions of each patient that showed several symptoms of *E. coli* infection. Then, the isolates were transported to our laboratory and stored in 10 % skim milk at –80°C until use.

Antimicrobial susceptibility testing: The following 12 antimicrobial agents were tested: ampicillin (ABPC), cefazolin (CEZ), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), colistin (CL), oxytetracycline (OTC), chloramphenicol (CP), bicozamycin (BCM), nalidixic acid (NA), enrofloxacin (ERFX), and trimethoprim (TMP). The MICs were determined by an agar dilution method according to the guidelines of the NCCLS [14]. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC29213, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 were used as quality control in MIC determinations. Breakpoints established by the NCCLS were used for the following 6 antimicrobial agents: ABPC, CEZ, KM, GM, CP, and ERFX [14]. The breakpoints for other antimicrobial agents were set as the midpoint between the peaks when the MICs were bimodally distributed.

PCR: Multiplex PCR for detection of the *stx1, stx2, hlyA*, and *eaeA* genes was performed using the primer sets reported by Fagan *et al.* [7]. The *stx2e* gene was detected in isolates that harbored the *stx2* gene with the PCR protocol reported by Johnson *et al.* [9].

PCR amplified products were analyzed by gel electrophoresis using 2% (wt/vol) agarose gel (Takara Shuzo Co., Ltd., Japan) in 1 × TAE buffer at 100 V. Gels were stained

with ethidium bromide, visualized with ultraviolet irradiation, and imaged with a GelDoc fluorescent imaging system (Bio-Rad, U. S. A.).

Statistical analysis: The χ^2 test was used for statistical analysis of the difference in resistant frequencies. Significance levels of 1% and 5% were used.

RESULTS

The MIC distribution of the 12 antimicrobial agents for the 175 *E. coli* isolates is shown in Table 1. Resistance to OTC was found most frequently (78.3%), followed by DSM (70.3%) and ABPC (49.1%). Comparing cattle and pigs, resistance to CL was more prevalent in pigs than in cattle ($p<0.01$), while resistance to CEZ was more prevalent in cattle than in pigs ($p<0.01$) (Fig. 1).

Of the 118 *E. coli* isolates from sick pigs, 59 harbored the *stx2e* gene, one harbored the *stx1* gene, and one harbored both the *stx1* and *stx2e* genes respectively. No porcine isolates harbored either the *hlyA* or *eaeA* genes. Resistance rates were significantly higher in STEC (n=61) than in non-STEC (n=57) for CL ($p<0.01$) and ERFX ($p<0.05$) (Fig. 2). On the other hand, of the 57 bovine with *E. coli*, 13 harbored the *stx1* gene, two harbored the *stx2* gene, and three harbored both the *stx1* and *stx2* genes, while no isolates har-

bored the *stx2e* gene. In contrast to the pigs, there were no significant differences in antimicrobial resistance rates between STEC (n=18) and non-STEC (n=39) isolates from sick cattle. Of the 18 bovine with STEC, 11 harbored the *hlyA* gene and 13 harbored the *eaeA* gene. There were no differences in antimicrobial resistance rates between the isolates with and without the genes.

DISCUSSION

In this study, the antimicrobial resistance rates of sick cattle and pigs were compared with those of apparently healthy animals as previously reported [10]. Resistance rates to all agents, except for BCM in cattle, were significantly higher in isolates from sick animals than those from healthy animals (Table 1). The similar tendency of *E. coli* from both origins was observed in the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DAN-MAP) report [8]. Half or more of the isolates from sick animals exhibited resistance to ABPC, DSM, and OTC, which were commonly used for treatment of respiratory and diarrhea diseases in the cattle and pig industries. The spread of these antimicrobial resistances illustrates the importance of drug choice on the basis of their antimicrobial susceptibility tests.

Table 1. The MIC distribution of antimicrobial agents for *E. coli* from sick cattle (n=57) and pigs (n=118)

Antimicrobial agents	MIC (mg/l)															Breakpoint (mg/l)	No. of resistant isolates (%)		
	<0.125	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512		Total	Cattle	Pigs
ABPC†				1	3	69	13	3			3	1	2	27	53	32a)	86 (49.1)	34 (58.6)**	52 (44.1)**
CEZ				65	52	31	12	1		7	2	5				32a)	14 (8.0)	9 (15.5)**	5 (4.2)**
DSM				10	26	6	5	5		9	18	28	31	16	21	32b)	123 (70.3)	44 (75.8)**	79 (66.9)**
KM				1	25	68	19	4	1	1		3	8	8	37	64a)	56 (32.0)	22 (37.9)**	34 (28.8)*
GM	1		14	88	44	2	2	2	1	8	7	4	2			16a)	22 (12.6)	5 (8.6)**	17 (14.4)**
CL			22	49	55	5	25	18	1							2b)	49 (28.0)	7 (12.1)**	42 (35.6)**
CP						14	44	41	5	9	9	6	25	17	5	32a)	71 (40.6)	20 (35.1)**	51 (43.2)**
OTC			1	1	29	6	1			1	15	100	17	4		16b)	137 (78.3)	42 (72.4)**	95 (80.5)**
BCM								4	69	88	4	1	7	2		128b)	10 (5.3)	1 (1.7)	9 (7.6)**
NA					4	42	53	5	7	6	5	11	11	3	28	64b)	58 (33.1)	17 (29.3)**	41 (34.7)**
ERFX	112	9	15	8	4	7	6	8	4	2						4a)	20 (11.4)	6 (10.3)**	14 (11.9)**
TMP	1	1	16	40	35	4	5	2	2						69	128b)	69 (39.4)	18 (31.6)**	51 (43.2)**

†: Ampicillin (ABPC), cefazolin (CEZ), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), colistin (CL), chloramphenicol (CP), oxytetracycline (OTC), bicozamycin (BCM), nalidixic acid (NA), enrofloxacin (ERFX), and trimethoprim (TMP).
a) : The value was a NCCLS breakpoint.
b) : The value was set as the midpoint between the peaks of each MIC distribution.
*,**: There were significant differences between resistance rates of isolates from sick animals and *E. coli* isolates from healthy animals reported by Kijima-Tanaka *et al.* (2003) that showed the following results: 8.4% (ABPC), 0.0% (CEZ), 20.8% (DSM), 3.4% (KM), 0.0% (GM), 3.1% (CL), 3.1% (CP), 25.3% (OTC), 0.6% (BCM), 2.0% (NA), 0.3% (ERFX) and 2.2% (TMP) in cattle; and 22.6% (ABPC), 0.0% (CEZ), 43.0% (DSM), 19.0% (KM), 2.8% (GM), 0.8% (CL), 22.3% (CP), 66.8% (OTC), 2.2 % (BCM), 0.8% (NA), 0.0% (ERFX), and 13.1% (TMP) in pigs (*: $p<0.05$, **: $p<0.01$).
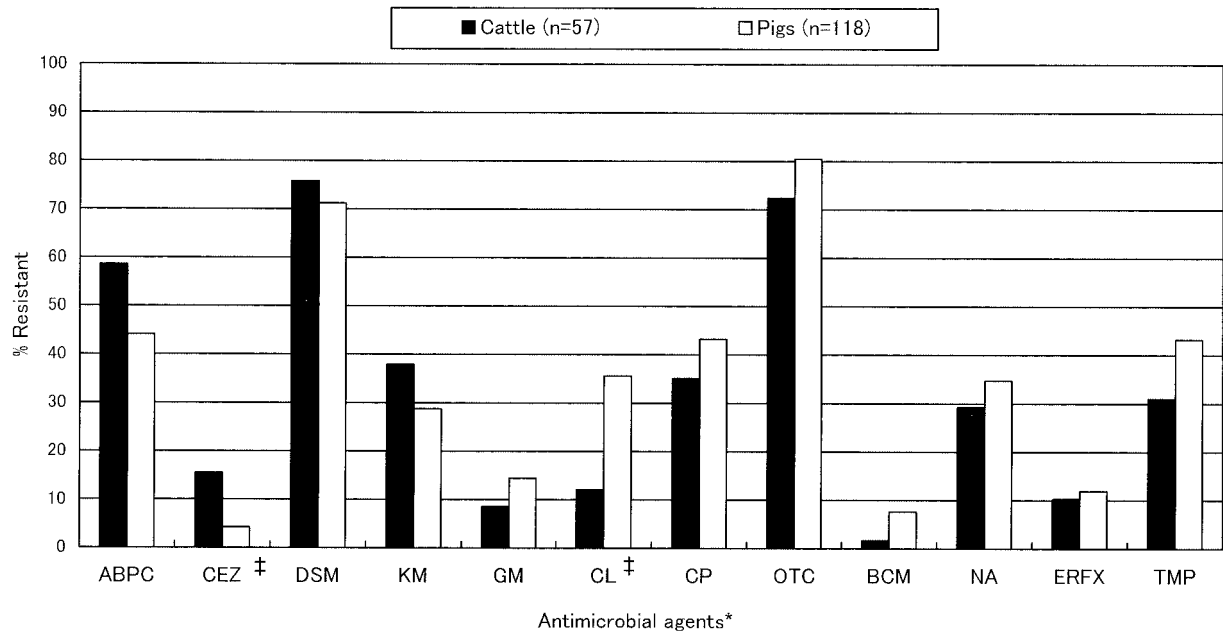
Fig. 1. Comparison of *E. coli* isolates from sick cattle and pigs. *: Ampicillin (ABPC), cefazolin (CEZ), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), colistin (CL), chloramphenicol (CP), oxytetracycline (OTC), bicozamycin (BCM), nalidixic acid (NA), enrofloxacin (ERFX), and trimethoprim (TMP). ‡: There were significant differences in antimicrobial resistance rates between *E. coli* isolates from cattle and pigs ($p<0.01$).
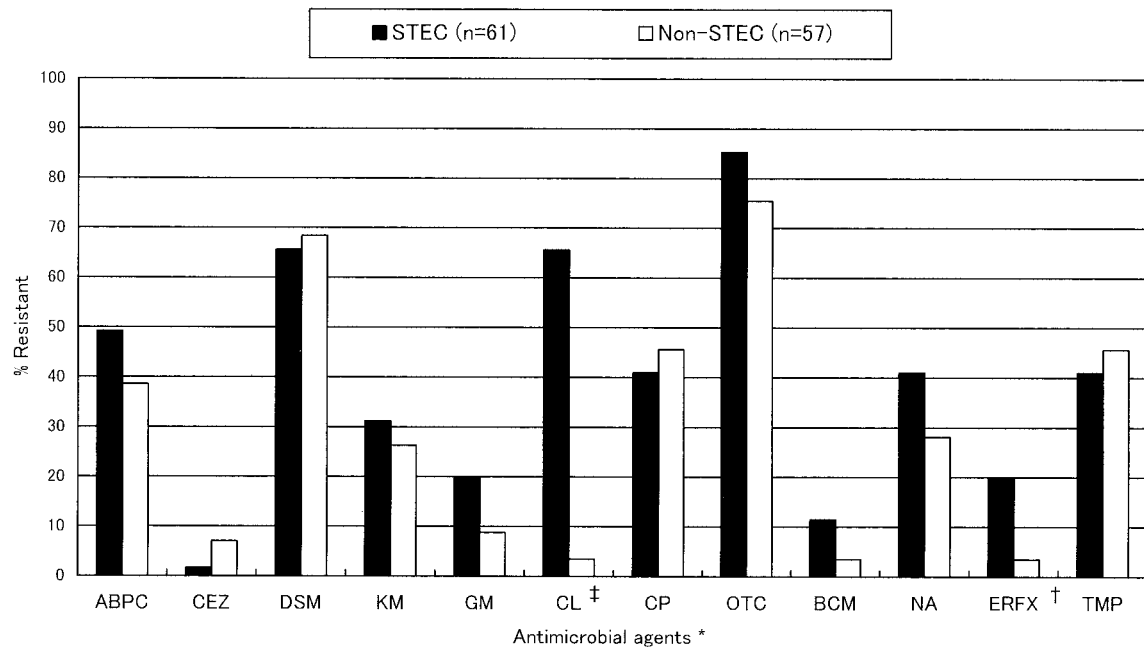

Fig. 2. Comparison of STEC and non-STEC isolates from sick pigs. *: Ampicillin (ABPC), cefazolin (CEZ), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), colistin (CL), chloramphenicol (CP), oxytetracycline (OTC), bicozamycin (BCM), nalidixic acid (NA), enrofloxacin (ERFX), and trimethoprim (TMP). †, ‡: There were significant differences in antimicrobial resistance rates between STEC and non-STEC isolates (†: $p<0.05$, ‡: $p<0.01$).

Comparing bovine and porcine isolates as a whole, there were significant differences in resistance to CEZ and CL (Fig. 1). Currently, CEZ is approved for cattle, but not for pigs. Conversely, CL is used much more for pigs than for cattle. Thus, high resistance rates originating in sick animals may be associated with the amount of antimicrobials used for each animal. On the other hand, CP resistant isolates remained more frequently in the isolates from sick animals than those from healthy animals in spite of banning the use of CP for livestock in Japan in 1998 [10]. Between 1998 and 1999 in the United States and 1995 and 2000 in Canada, CP resistant isolates remained in pathogenic *E. coli* isolates from pigs after banning the use of CP in food animals in the 1980's [2, 11]. Co-selection by the use of other antimicrobial agents may contribute to the higher prevalence of CP resistance originating in sick animals [2, 11]. Three or more antimicrobial-resistant *E. coli* isolates accounted for 97% and 47% of CP resistant and susceptible isolates (data not shown). Though the resistance mechanisms, such as integron, transposon, and plasmid, are known [3, 13, 16, 21], little information about their prevalence in domestic animals in Japan is available. Therefore, these results suggested that the resistance frequencies reflected, in part, on the present conditions of antimicrobial agents administered for treatment against *colibacillosis*.

In our investigation about the virulence factors, the prevalence of isolates that harbored the *stx1* or *stx2e* gene from sick pigs was associated with antimicrobial resistances, while there were no significant differences in sick cattle. It is considered that the result in cattle suggests that common antimicrobial agents are administered for cattle without any distinction for virulence factors. On the other hand, there were significant differences in resistance to CL and ERFX between STEC and non-STEC isolates in pigs (Fig. 2). Choi *et al.* [6] reported that there were no significant differences in the antimicrobial resistance of pathogenic *E. coli* from sick pigs in Korea, but the report did not examine the two antimicrobials. It may be clarified that STEC isolates from pigs are exposed to their selective pressures of them if a study concerning resistance mechanisms to them proceeds. ERFX is approved for colibacillosis in pigs in Japan, but is not considered the first choice drug because it is important for humans. On the other hand, CL is one of the first choice medicines for edema disease in pigs. As CL is used not only as a medicine but also as a feed additive, CL used as a feed additive may commonly affect bacteria in healthy and sick animals.

Sulfamethoxazole/TMP, as well as CL, is widely used for treatment against colibacillosis as a first choice drug. CL resistance is scarcely acquired [4,15]. A breakpoint concentration of 16 mg/*l* is recommended for CL by DANMAP, but the test range for CL was from 2 to 64 mg/*l* [8]. As the MIC of CL showed a bimodal distribution, ranging from 0.25 to 16 mg/*l*, the breakpoint for CL was determined to be 2 mg/*l* based on the distribution of the MIC in our study. There was little difference between the MIC of CL susceptible and resistant isolates. On the other hand, all of the 69 TMP resistant strains exhibited very high MICs (>512 mg/*l*). These results suggest that an antimicrobial susceptibility test before treatment and observation of clinical symptoms after treatment are necessary when TMP for colibacillosis.

In conclusion, antimicrobial resistance is more prevalent in *E. coli* isolates from sick animals than apparently healthy animals. There was a difference in prevalence of antimicrobial resistance between STEC and non-STEC in pigs, but not in cattle. These results can be considered helpful for adequate selection and prudent use of antimicrobial agents for colibacillosis.

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