ATCC 35218, *E. coli* NIHJ, *Staphylococcus aureus* ATCC 25923, *S. aureus* 209P and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control. Breakpoints were those described previously (Kijima-Tanaka et al., 2003).

Results

Isolation of STEC

A total of 609 faecal samples (272 from cattle, 179 from pigs and 158 from broilers) were collected from 596 farms of all 47 prefectures across Japan. STEC were isolated from 62 bovine faecal samples (23%) and 25 swine samples (14%). Three of the positive bovine samples had different serotypes or *stx* types of STEC, so we chose two isolates each from these three samples. Finally, we chose 65 bovine isolates and 25 swine isolates for further study. Of these isolates, 23 bovine STEC were isolated in prefecture laboratories and the remainder were isolated in our laboratory. These isolates originated from a wide area of Japan: the bovine isolates from 32 prefectures and the swine ones from 17 prefectures. Overall, bovine and swine STEC were present in all eight districts (Hokkaido-Tohoku, Kanto, Chubu, Tokai, Kinki, Chugoku, Shikoku and Kyushu-Okinawa) of Japan. No STEC was found in the 158 broiler faecal samples.

Virulence-associated characterization of STEC

Serotypes and other virulence-associated characteristics of STEC are shown in Table 2. For the bovine isolates, 65 isolates could be classified into 32 serotypes. Among them, nine isolates belonged to the serogroups of O157 and O26, which are most frequently detected as causal agents of human diseases. All of these isolates possessed the *eae* genes; *eae-γ1* for all O157, and *eae-β1* for all O26 isolates. Three isolates belonged to the O113:H21 serotype and seven to O145:NM, which serotypes were also frequently implicated in human diseases. *Eae* gene was not detected from O113:H21 isolate, and *stx* and *eae* genes including *eae*-subtypes were varied in O145:NM isolates (Table 2). For the other 46 bovine isolate, *stx2*-positive, *eae*-negative isolates were dominant (25 isolates). *Eae*-genes were observed for 13 isolates, of these, one was *eae-β1* of O103:H11 isolate, and the other 12 were not *eae-β1* nor *eae-γ1*. Geographically, two isolates of O157:H7, two isolates of O84:NM and four isolates of O174:H21 were derived from different farms in the same prefecture, and for the other isolates of identical serotypes, the geographical origins were from far-spaced prefectures, each other.

The swine isolates belonged to 15 serotypes, including three not typeable isolates. The serotypes and other virulence-factor genotypes were quite different from those of the bovine isolates. Serotypes of frequently implicated in human diseases, or *eae*-positive isolates were not observed in swine isolates. *Stx* type was *stx2* in 24 of 25 isolates, and many of them (18 isolates) were *stx2e*, which was associated with oedema diseases in pigs (DeGrandis et al., 1989). For the six O100:NM isolates from five far-spaced prefectures, *stx2* type was not *stx2e*.

Anti-microbial resistance

The MICs of the STEC isolated are shown in Table 3. MIC distributions range, MIC_{50} (MIC values that inhibit 50% of the isolates tested) and MIC_{90} (MIC values that inhibit 90% of the isolates tested) and rates of resistance were generally higher in swine isolates than in bovine ones. High rates of resistance were observed against sulphadimethoxine (68% pigs and 55% cattle), oxytetracycline (64 and 32%) and dihydrostreptomycin (44 and 31%), followed by ampicillin and kanamycin (11–16%). For chloramphenicol, higher rates of resistance were observed among the swine isolates (48%) but low rates among the bovine isolates (3%). Trimethoprim resistance showed a similar tendency. Rates of resistance to the other anti-microbials were ≤4%. Resistance to cephalosporins (cefazolin, cefuroxime and ceftiofur), quinolones (nalidixic acid, oxolinic acid, enrofloxacin, and ofloxacin) and bicozamycin was not observed.

Anti-microbial resistance patterns of STEC are shown in Table 4. Twenty-seven bovine isolates (42%) were susceptible to all 18 anti-microbials assayed (data not shown). Fifteen isolates (23%) showed single resistance and 23 (35%) showed multiple resistance. Among the multi-resistant isolates, six (O26:H11, O26:NM, and four isolates of O145: NM) were serotypes frequently implicated in human disease. Multiple resistance was also frequently observed in O2:NM and O174:NM/H21 isolates: among these, four O2:NM isolates from far-spaced prefectures, and three O174:H21 isolates from different farms in the same prefecture and one O174:H21 isolate from far-spaced prefectures showed identical resistance patterns. The most frequent resistance pattern of the bovine isolates was sulphadimethoxine–oxytetracycline–dihydrostreptomycin resistance, and additional patterns of resistance to kanamycin and/or ampicillin were also frequently observed.

Fifteen of the 25 swine isolates (60%) showed multiple resistance and seven (28%) showed single resistance (Table 4). Only three isolates (12%) were susceptible to all anti-microbials. The swine isolates showed various resistance patterns including resistance to as many as seven anti-microbials. The resistance patterns were different from those of the bovine isolates, and patterns including chloramphenicol were more common than with the bovine isolates.

Discussion

In Japan, rates of human STEC infection abruptly increased in 1996 because of a large-scale outbreak of O157 infection in Sakai city: government records of STEC isolation from human cases increased to 3022 in that year and have been kept at about 2000 a year since then (http://idsc.nih.go.jp/iasr/index.html). O157:H7 was the dominant serotype in those cases, followed by O26:H11. And about 90% of human isolates belonged to serogroups O157 and O26. In our study, a total of nine of bovine isolates belonged to O157 or O26, and all of these isolates possessed the *eae* genes of *eae-γ1* for O157, and *eae-β1* for O26 isolates. Special attention should be given to these isolates to follow their rates of prevalence and anti-microbial resistance. We also detected three O113:H21 isolates and seven O145:NM isolates in cattle; these isolates were also frequently implicated in human cases followed by O157 or O26. Our O113:H21 isolates lacked the *eae* gene, and this is commonly observed in the literature on O113:H21 strains from human disease (Gyles et al., 1998; Paton et al., 1999). Further studies of virulence factors such as $lpfA_{O113}$ (Doughty et al.,

Table 2. Virulence-associated characteristics of STEC isolated from food-producing animals in Japan

Source	Serotype[a]	No. of isolates	No. of isolates with the following genes[b]: *stx1*	*stx2*	*stx1* + *stx2*	*eae*
Cattle	**O157:H7**	3	1	1	1	3 (*γ1*)
	O157:H NT	1	0	1	0	1 (*γ1*)
	O26:NM	2	2	0	0	2 (*β1*)
	O26:H11	1	0	0	1	1 (*β1*)
	O26:H21	1	1	0	0	1 (*β1*)
	O26:H NT	1	1	0	0	1 (*β1*)
	O113:H21	3	0	3	0	0
	O145:NM[c]	7	3	4	0	2 (*β1*(1), *γ1*(1))
	O1:H45	1	0	1	0	0
	O2:NM	7	0	7	0	1
	O8:NM	1	0	1 (*stx2e*)	0	0
	O15:H NT	1	0	1	0	0
	O22:H NT	3	0	1	2	0
	O46:H NT	1	0	0	1	0
	O46:H11	1	0	0	1	0
	O74:NM	1	1	0	0	1
	O84:NM	5	5	0	0	5
	O88:NM	1	0	1	0	0
	O98:NM	1	1	0	0	1
	O103:H11	1	1	0	0	1 (*β1*)
	O113:H11	1	0	0	1	0
	O113:H19	2	0	2	0	0
	O113:NM	2	0	1	1	0
	O116:NM	1	0	1	0	0
	O119:H16	1	0	1	0	0
	O119:NM	2	2	0	0	2
	O146:NM	1	1	0	0	0
	O165:NM	2	1	0	1	2
	O174:H21	6	0	6	0	0
	O174:NM	2	0	2	0	0
	NT:H NT	1	0	1	0	0
	Rough:NM	1	1	0	0	0
	Total[d]	65	21 (32.3)	35 (53.8)	9 (13.8)	24 (36.9)
Pigs	O2:NM	2	0	2 (*stx2e*)	0	0
	O2:H NT	1	0	1 (*stx2e*)	0	0
	O8:H19	1	0	1 (*stx2e*)	0	0
	O91:H NT	1	1	0	0	0
	O100:NM	6	0	6	0	0
	O100:H NT	1	0	1 (*stx2e*)	0	0
	O104:NM	1	0	1 (*stx2e*)	0	0
	O121:NM	2	0	2 (*stx2e*)	0	0
	O121:H10	2	0	2 (*stx2e*)	0	0
	O123:H NT	3	0	3 (*stx2e*)	0	0
	O141:H4	1	0	1 (*stx2e*)	0	0
	O141:H NT	1	0	1 (*stx2e*)	0	0
	NT:NM	1	0	1 (*stx2e*)	0	0
	NT:H28	1	0	1 (*stx2e*)	0	0
	NT:H NT	1	0	1 (*stx2e*)	0	0
	Total	25	1 (4.0)	24 (96.0)	0 (0.0)	0 (0.0)

[a]NM, non-motile isolates. NT, not typeable isolates with O1 to O175 anti-sera, or with commercially available 22 H anti-sera. Bold and underlined serotypes are serotypes frequently implicated in human disease. Two isolates of O157:H7, two isolates of O84:NM and four isolates of O174:H21 were derived from different farms in the same prefecture, and for the other isolates of identical serotypes, the geographical origins were from widely separated prefectures.
[b]*Stx2e* gene was tested for *stx2*-positive isolate, *eae-β1* and *eae-γ1* genes were tested for eae-positive isolates. Number in parentheses is number of isolate, and no number means 'all' corresponding isolates.
[c]Virulence-factor genotypes of O145:NM isolates were as follows; three isolates of *stx2*, two isolates of *stx1*, one isolate each of *stx1-eae-γ1* and *stx2-eae-β1*.
[d]Numbers in parentheses are percentages of the total isolates from the respective sources.

2002) might be required for these isolates. For the other 46 bovine isolates, *stx2*-positive and *eae*-negative STEC were mostly prevalent as 25 isolates. These results are consistent with those of earlier reports that *stx2*-positive, *eae*-negative STEC are dominant in healthy calves and might be components of their normal intestinal flora (Beutin et al., 1995; Blanco et al., 1997). And although *eae*-genes were observed for 13 isolates, *eae* subtype was not *eae-β1* nor *eae-γ1* except one O103:H11 isolate. Some reports indicated that human or bovine isolates of STEC O103 were closely associated with *eae-ε* (Oswald et al., 2000; Blanco et al., 2004), but our O103:H11 isolate reacted with *eae-β1* primers like O103:H2 isolates of

Table 3. MIC of STEC strains isolated from food-producing animals in Japan

Anti-microbial agent	Breakpoint[a] (mg l^{-1})	Bovine isolates (n = 65)			Swine isolates (n = 25)		
		MIC_{50}[b] (mg l^{-1})	MIC_{90}[b] (mg l^{-1})	No. of resistant isolates (%)	MIC_{50} (mg l^{-1})	MIC_{90} (mg l^{-1})	No. of resistant isolates (%)
Ampicillin	12.5	3.13	25	7 (10.8)	3.13	> 50	4 (16.0)
Cefazolin	25	1.56	1.56	0 (0.0)	1.56	1.56	0 (0.0)
Cefuroxime	50	3.13	3.13	0 (0.0)	3.13	6.25	0 (0.0)
Ceftiofur	3.13	0.39	0.39	0 (0.0)	0.39	0.78	0 (0.0)
Dihydrostreptomycin	50	3.13	> 100	20 (30.8)	25	> 100	11 (44.0)
Kanamycin	12.5	1.56	> 100	8 (12.3)	1.56	> 100	4 (16.0)
Gentamicin	3.13	0.39	0.78	0 (0.0)	0.39	0.78	1 (4.0)
Apramycin	12.5	1.56	3.13	0 (0.0)	3.13	3.13	1 (4.0)
Colistin	1.56	0.39	0.78	2 (3.1)	0.39	0.78	0 (0.0)
Chloramphenicol	50	6.25	6.25	2 (3.1)	25	> 100	12 (48.0)
Oxytetracycline	12.5	3.13	> 100	21 (32.3)	100	> 100	16 (64.0)
Bicozamycin	100	25	25	0 (0.0)	25	25	0 (0.0)
Nalidixic acid	50	3.13	3.13	0 (0.0)	3.13	12.5	0 (0.0)
Oxolinic acid	12.5	0.2	0.39	0 (0.0)	0.39	3.13	0 (0.0)
Enrofloxacin	3.13	≦ 0.05	≦ 0.05	0 (0.0)	≦ 0.05	0.2	0 (0.0)
Ofloxacin	3.13	0.1	0.1	0 (0.0)	0.1	0.2	0 (0.0)
Trimethoprim	12.5	0.2	0.39	2 (3.1)	0.39	> 50	3 (12.0)
Sulphadimethoxine	400	400	> 400	36 (55.4)	> 400	> 400	17 (68.0)

[a]Breakpoints are those described by Kijima-Tanaka et al. (2003).
[b]MIC_{50} is MIC values that inhibit 50% of the isolates tested, and MIC_{90} is MIC values that inhibit 90% of the isolates tested.

rabbit origin (Oswald et al., 2000), and did not react with *eae*-ε primers of Blanco et al. (2004) (data not shown).

In contrast with the bovine isolates, serotypes frequently implicated in human disease or *eae*-positive isolates were not observed in swine isolates. Many of swine isolates possessed the *stx2e* subtype. Stx2e has been referred to as 'pig oedema disease toxin', and has been demonstrated not share to the same glycolipid-binding specificity with Stx2 (DeGrandis et al., 1989). *Stx2* subtype of six O100:NM isolates was not *stx2e*, but none of these isolates had *eae* genes. These findings suggested that our swine isolates were genetically not closely associated with human isolates. Similarly, DesRosiers et al. (2001) suggested that swine and human isolates in Canada are not genetically related. However, Nakazawa et al. (1999) reported the first isolation of STEC O157:H7 from pigs in Japan, and continuous monitoring of swine STEC might be necessary.

STEC were isolated from 23% of bovine faecal samples and 14% of swine faecal samples over a wide area of Japan. As for the STEC detection methods other than serogroup of O157, effective methods have not been widely reported; and the use of different detection methods and different sampling design make it difficult to compare STEC prevalence rates reported for various geographic areas. In previous Japanese reports, however, Miyao et al. (1998) had a 24% STEC isolation rate from cattle at a slaughterhouse of central prefecture, Tokyo, Fukushima and Seki (2004) had a 15.9% STEC isolation rate from cattle at a slaughterhouse of western prefecture, Shimane, and Kobayashi et al. (2001) had STEC isolation rates of 19–31% from healthy cattle in central Japan, and our bovine data appear comparable with these. Considering that the real 'STEC prevalence rate' would be higher than the 'STEC detection rate', our data indicate that STEC are spread generally throughout Japan. STEC isolation reports on healthy swine are limited, but our level of 14% is higher than those in Germany (7.5%; Beutin et al., 1993) and Hong Kong (2.1%; Leung et al., 2001) and shows that pigs are also potential reservoirs of STEC in Japan. STEC were not isolated from the 158 broiler samples. Some reports indicated that colonization and faecal shedding of STEC were observed in chicks after oral challenge (Stavric et al., 1993; Schoeni and Doyle, 1994; La Ragione et al., 2005), and STEC were detected from 'poultry meat of grocery stores' (Doyle and Schoeni, 1987; Samadpour et al., 1994). STEC was not, however, detected from chicken faecal samples at farms or slaughterhouse (Beutin et al., 1993; Chapman et al., 1997; Kobayashi et al., 2002; Wani et al., 2004) or was detected at a low level (1%; Cobeljic et al., 2005). Considering these, chickens might not be potential reservoirs of STEC.

Anti-microbial resistance was higher in the swine isolates than in the bovine ones. Under JVARM, we also monitored the anti-microbial resistance of *E. coli* as indicator bacteria in a total of 1018 isolates (Kijima-Tanaka et al., 2003), revealing again that resistance was more frequent among swine isolates than bovine isolates. Our STEC results might reflect the resistance situation of *E. coli* in general. We observed high rates of resistance to sulphadimethoxine, oxytetracycline and dihydrostreptomycin, followed by ampicillin and kanamycin. All of these anti-microbials are commonly used and classically approved in Japan. Chloramphenicol resistance was high in swine isolates but low in bovine isolates, possibly because chloramphenicol has been used in pigs but not in cattle in Japan. The use of chloramphenicol was banned in all animals except pets in Japan in 1998. However, persistently high rates of chloramphenicol-resistance have been reported years after withdrawal in Europe (Aalbaek et al., 1991) and the United States (Bischoff et al., 2002). Bischoff et al. (2002) suggest that this might have happened by co-selection of chloramphenicol resistance with either common virulence genes or other anti-microbial resistance phenotypes. This indicates that continuous monitoring is clearly also necessary for chloramphenicol resistance. Fluoroquinolones and the third generation cephalosporins are currently the most valuable anti-microbials in human medicine in Japan, and their veterinary use

Table 4. Anti-microbial resistance patterns of STEC

Source	No. of anti-microbials resisted	Resistance pattern[a]	No. of isolates	Serotype of STEC[b]
Cattle	1	SDM	14	**O157:H7** (2), **O157:H NT**, **O26:H21**, **O26:NM**, **O26:H NT**, **O145:NM**, O1:H45, O2:NM (2), 84:NM (2), O98:NM, O103:H11
		OTC	1	O84:NM
	2	SDM, OTC	1	O174:H21
		SDM, DSM	1	O8:NM
		SDM, ABPC	1	O174:H21
		SDM, CL	1	**O26:NM**
	3	SDM, OTC, DSM	6	**O145:NM** (*stx2*), O2:NM (4[c]), O174:NM
	4	SDM, OTC, DSM, KAN	5	O174:NM, O174:H21(4[d])
		SDM, OTC, DSM, AMP	1	O84:NM
		SDM, OTC, DSM, CHL	1	O113:H19
		OTC, DSM, AMP, CL	1	**O145:NM** (*stx1*)
	5	SDM, OTC, DSM, KAN, AMP	3	**O26:H11**, **O145:NM** (*stx2*, *eae-β1*), O116:NM
		SDM, OTC, DSM, CHL, TMP	1	**O145:NM** (*stx2*)
		SDM, OTC, DSM, AMP, TMP	1	Rough:NM
Pigs	1	SDM	3	O100:NM (2), O123:H NT
		OTC	2	O2:H NT, O121:H10
		DSM	2	O91:H NT, O100:H NT
	2	SDM, OTC	1	O121:NM
		SDM, DSM	1	NT:H28
	3	SDM, OTC, CHL	3	O2:NM, O121:NM, O123:H NT
		OTC, DSM, CHL	1	O100:NM
	4	SDM, OTC, CHL, TMP	1	O104:NM
		SDM, OTC, DSM, AMP	1	O100:NM
		SDM, OTC, DSM, CHL	1	O2:NM
	5	SDM, OTC, DSM, CHL, AMP	1	O100:NM
		SDM, OTC, DSM, CHL, KAN	1	O100:NM
		SDM, OTC, DSM, CHL, TMP	1	O121:H10
	6	SDM, OTC, CHL, KAN, AMP, TMP	1	O123:H NT
		SDM, OTC, DSM, CHL, KAN, AMP	1	O8:H19
	7	SDM, OTC, DSM, CHL, KAN, GEN, APM	1	NT:NM

[a]SDM, sulphadimethoxine; OTC, oxytetracycline; DSM, dihydrostreptomycin; CHL, chloramphenicol; KAN, kanamycin; AMP, ampicillin; TMP, trimethoprim; CL, Colistin; GEN, gentamicin; APM, apramycin.
[b]Numbers in parentheses are numbers of isolates. *Stx* and *eae* type were varied in O145:NM isolates, and were stated in parentheses for multi-resistant isolates of this serotype. Bold and underlied serotypes are serotypes frequently implicated in human disease. NM, non-motile isolates. NT, not typeable isolates with O1 to O175 anti-sera, or with commercially available 22 H anti-sera.
[c]Four O2: NM isolates were derived from far-spaced prefectures.
[d]Three O174:H21isolates were derived from different farms in the same prefecture, and the fourth was from a distant prefecture.

has been restricted in Japan since their introduction to the veterinary market. It is therefore encouraging that we did not observe resistance to the quinolones, cephalosporins and bicozamycin.

Anti-microbial resistance patterns differed between bovine and swine isolates. Multi-resistant STEC were relatively few among the bovine isolates (23 isolates; 35%), but six of them belonged to the serotypes frequently implicated in human disease (O26:H11, O26:NM and four O145:NM isolates). Hiruta et al. (2000) reported an outbreak of diarrhoea due to multiple-resistant O26:H11; that resistance was encoded by a transmissible plasmid, and that resistance pattern was same as our O26:H11 isolate. Yamamoto and Wakisaka (1998) reported that resistance was more frequently observed in O26 than in O157 among human STEC. We had the same finding in our study of bovine STEC, and careful monitoring of anti-microbial resistance in O26 and O145:NM is therefore needed.

Among the bovine isolates, the prevalent resistance pattern was that of sulphadimethoxine–oxytetracycline–dihydrostreptomycin. This resistance pattern has been reported in the United States as the most frequent pattern of resistance in O157:H7 of human and bovine origin (Kim et al., 1994; Meng et al., 1998). Yamamoto and Wakisaka (1998) also reported that this resistance pattern was prevalent within STEC of human and bovine origin in Japan. Our present data support these reports, and may demonstrate the similarity between human and bovine isolates. We observed additional patterns of resistance to kanamycin and/or ampicillin in our bovine isolates, including serotypes O26:H11 and O145:NM; such patterns are also frequently observed in human STEC. Furthermore, we observed multiple resistance in O2:NM and O174:NM/H21 isolates. Data on the resistance patterns of STEC other than O157 or O26 are limited, and it is noteworthy that four of our seven bovine O2:NM isolates from distant prefectures showed identical resistance patterns. Among the swine isolates, the resistance patterns varied. Resistance patterns frequently observed in human isolates, such as sulphadimethoxine–oxytetracycline–dihydrostreptomycin, were not observed in swine isolates. None of the swine isolates had serotypes of frequently implicated in human disease, and although resistance was observed more frequently in swine isolates, resistance to the quinolones and cephalosporins was not observed. Therefore, there may be only a low

possibility that these swine STEC will become hazardous to humans.

This study is the first national survey of STEC in the veterinary field across Japan. Anti-microbial use in animals, as in humans, inevitably selects for resistant bacteria, which could be transmitted to humans via food, and this is a matter of public health concern. To minimize the negative public health impact of the use of anti-microbials in food-producing animals, appropriate and prudent use of anti-microbials and continuous monitoring should be considered in the future.

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