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Characterization of multidrug-resistance phenotypes and genotypes of *Escherichia coli* strains isolated from swine from an abattoir in Osaka, Japan

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SUMMARY

A total of 455 highly tetracycline-resistant *Escherichia coli* strains were isolated from 84 healthy swine from abattoirs and it was found that 56·9, 43·1, 22·2, 15·4, 2·6 and 1·5% of strains were resistant to chloramphenicol, ampicillin, kanamycin, trimethoprim–sulphamethoxazole, ofloxacin and gentamicin respectively. Interestingly, *E. coli* strains isolated from certain finisher hog groups exhibited resistance against 2–7 antimicrobials, but strains isolated from multiparous sow groups in each herd were resistant to only 2–4 antimicrobial agents. When randomly selected 108 tetracycline-resistant isolates were tested for the presence of resistance genes, the following genes *tet*(A) ($n=6$), *tet*(B) ($n=95$), *tet*(D) ($n=1$) or both *tet*(A) and *tet*(B) ($n=6$) were found to be distributed among them. Furthermore, 52 isolates carried the integrase 1 gene and 24 strains gave five different PCR amplicon profiles using primers from the variable region of integron. Extensive nucleotide sequence analyses of these amplicons revealed the presence of *dhfrI*, *dhfrXII*, *dfr17*, *aadA*, *aadA2*, *aadA5*, *aadA21*, *aacA4* and *catB3* genes which code for different antibacterial resistance proteins.

INTRODUCTION

On the basis of rapid development of intensive production systems for food-producing animals, various antibmicrorial agents are often widely used in livestock [1]. Antimicrobials are mainly used in animals for four different purposes, e.g. (i) therapeutic, (ii) metaphylactic, (iii) prophylactic, and (iv) growth promotion [2, 3] or food additives. The European federation for animal health (FEDESA) [4] reported that the worldwide use of antibiotics for animal health purposes in 1996 was estimated at around 27 000 tons of which approximately 25% was used by the European countries alone. It was also estimated that 90% of all the antibiotics used for veterinary purposes are distributed mainly via animal feed [3, 5]. Animal-wise distribution of antibiotics was: pigs (60%), poultry and rabbits (20%), ruminants (18%), fish (1%), and pets (1%) [3]. FEDESA [4] also reported that the total sales volume of antibiotics in

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the European Union plus Switzerland in 1997 was 3494 tons of drugs used for therapy but not for growth promotion in animals which included tetracyclines (2294 tons), macrolides (424 tons) and penicillins (322 tons) [2–4]. The Japanese Ministry of Agriculture, Forestry and Fisheries reported that in 2001 the total sales volume of antimicrobial agents for animal health purposes in Japan was 1059 tons including tetracyclines (456 tons), sulphonamides group (175 tons) and macrolides (161 tons) [6]. It was estimated that they were mainly used for pigs (54%), fish (20%), broiler chickens (11%), and cattle and layer (4%) [6].

In veterinary medicine, the old-generation antibiotic tetracyclines have widely been used throughout the world because they are broad-spectrum in nature and have growth-inhibitory activity to atypical organisms such as Chlamydiae, certain mycoplasmas, Rickettsiae and protozoan parasites, in addition this group of drugs are relatively safe, less expensive and less toxic [3, 7–9]. It has also been pointed out, however, that use of antimicrobials for food-producing animals may be a risk to public health due to the presence of residual drugs in animal products and/or the spread of drug-resistant bacterial strains [2, 5, 10]. In Japan, tetracyclines are the most commonly used antibiotics in animals [6] and they have been detected as residues in carcasses of slaughtered swine [11, 12]. Furthermore, in Japan swine breeding is advancing at a large-scale and in intensive form compared to that of cattle, sheep, horses or goats. Although the number of swine-breeding farms have declined steadily from 530 000 to 11 000 from 1955 to 2001 [13, 14], within the same time span the number of average swine-breeding herds has increased from 1·6 to 906 [13, 14] and this tendency appears to be maintained steadily. It was thought that these changes possibly enhance the use of antimicrobials for food-producing animals, to protect the animals from infectious diseases and increase productivity, which may result in the emergence of multidrug-resistant bacterial strains.

In this study, we isolated highly tetracycline-resistant *E. coli* strains from healthy swine from an abattoir in Osaka, Japan and these strains were further examined for multidrug resistance against 11 antimicrobials which are used for humans. Furthermore, the class 1 integron was detected in the genomes of *E. coli* strains. The role of this gene-capturing cassette to multidrug resistance is also discussed.

MATERIALS AND METHODS

Selection of swine herds

Ten swine herds were selected on the basis of the location of their farms and age groups, namely approximately 6 months old, and named finisher hog group, and multiparous sow group. The swine farms were located more than 40 km apart from each other. Ten healthy finisher hogs were randomly selected from each farm. All the healthy multiparous sows were selected from each farm since their numbers were always less than 10 (Table 1).

Collection of swine faecal samples

Before collection of faecal samples from swine, it was confirmed by interviewing the farmers that the animals had not been given any antimicrobials for therapeutic purposes. Faecal samples from healthy swine of finisher hog groups and multiparous sow groups were collected aseptically from their rectum after slaughtering at the Osaka Prefecture abattoir in February 2001. Collected faecal samples were immediately placed on ice and transported to the laboratory for bacteriological study.

Isolation of highly tetracycline-resistant *E. coli* strains

To isolate tetracycline-resistant *E. coli* strains, 5 g of each faecal sample was suspended in 45 ml of sterile saline (0·9% NaCl, w/v), followed by ten-fold serial dilution and plating of 100 μl of each dilution on MacConkey agar (Difco Laboratories, Detroit, MI, USA) containing 256 μg/ml of tetracycline (Wyeth Lederle Japan Ltd, Tokyo, Japan). After incubation of the inoculated plates at 37 °C for 18–24 h, 10 lactose-fermenting (LF) colonies were randomly selected from a plate, or at least 10 LF colonies per faecal sample, or if in any sample the number of colonies were less than 10 then all the available colonies were selected. Each of the colonies selected from plates was identified as *E. coli* by using the ID test EB-20 (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan). If needed, 16S rRNA DNA fragment was PCR amplified from the bacterial genome and the amplicon was directly sequenced to confirm it as *E. coli*. *E. coli* strains with a MIC value greater than 256 μg tetracycline/ml determined by using the Etest (AB Biodisk, Solna, Sweden) were considered resistant. Briefly, each isolate was diluted to achieve turbidity of a 0·5 McFarland's standard manually,

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Table 1. *Estimated number of highly tetracycline-resistant faecal* E. coli *strains isolated from swine from different farms*[a]

Herd no.	Location of farm in different prefectures[b]	Age group[c]	No. of positive/ tested no.	No. of resistant strains[d]	Estimated no. of resistant strains (c.f.u./g of faeces)
1	Mie, A	F	10/10	100	10^6–10^7
2	Kyoto	F	10/10	100	10^4
3	Tottori, A	F	5/10	18	$<10^2$–10^3
4	Tottori, B	F	8/10	43	$<10^2$–10^4
5	Osaka, A	F	10/10	54	10^2–10^4
6	Wakayama	F	0/10	0	$<10^2$
7	Nara, A	F	0/10	0	$<10^2$
8	Mie, B	S	2/2	20	10^4
9	Nara, B	S	4/4	40	10^5
10	Osaka, B	S	8/8	80	10^5
Total			57/84	455	

[a] Highly tetracycline resistant was defined when MIC of the drug was $>256\,\mu$g/ml.
[b] The swine farms were located more than 40 km apart from each other, A and B indicate two different farms in the same prefecture.
[c] F, Finisher hogs approximately 6 months old group; S, multiparous sows group.
[d] Ten isolates were tested for sensitivity against tetracycline per swine.

inoculated onto Mueller–Hinton agar (Difco Laboratories) plates with a depth of $4{\cdot}0 \pm 0{\cdot}5$ mm. The Etest strip was manually applied in the centre of the plate within 15 min and the plate was incubated at 35 °C for 18 h. After incubation, the MIC value was determined to read the point of intersection between the inhibition ellipse edge and the Etest strip.

Antimicrobial susceptibility test

A battery of antimicrobial agents which are in use for humans especially for Gram-negative bacteria were selected. The MICs of these drugs were determined as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for antimicrobial disc diffusion assay [15]. Initially the antimicrobial sensitivity of *E. coli* isolates was screened by using commercially available discs (Eiken Chemical Co. Ltd, Tokyo, Japan) according to the instructions provided by the manufacturer. Methods of dilution of bacterial culture and inoculation of isolates on plates were the same as described above for the Etest. In a plate, 11 different antimicrobial discs namely, chloramphenicol, ampicillin, kanamycin, trimethoprim–sulphamethoxazole, ofloxacin, gentamicin, cefazolin, cefuroxime, cefotaxime, colistin, and imipenem were placed manually onto the plate and incubated at 35 °C for 18 h. Subsequently the isolates which showed intermediate or resistant inhibitory zones were analysed to determine their MICs quantitatively against the drugs using the Etest as described above. When the aminoglycoside-resistance gene was detected in the class 1 integron (see below), we examined retrospectively streptomycin resistance of *E. coli* strains according to Van den Bogaard et al. [16] using the Etest as described above. *E. coli* strain ATCC 25922 was always used as a reference strain.

Molecular biological methods

DNA templates for PCR analysis were prepared by the boiling method. Briefly, a colony was selected from the plate and suspended in 500 μl TE buffer [10 mM Tris–HCl, 1 mM EDTA (pH 8·0)], followed by incubation in boiling water for 5 min. The supernatant obtained after centrifugation at 9000 ***g*** for 5 min was used directly as a template DNA for PCR. The PCR reaction mix (total 50 μl) included 1 μl of template DNA solution, 1 × PCR buffer, 2·5 U LA-*Taq* or r*Taq* polymerase (Takara, Kyoto, Japan), 250 μM of each of the deoxynucleotides, dATP, dCTP, dGTP and dTTP, primers and distilled water. PCR conditions are described in Table 2. DNA amplification was carried out in a Takara PCR Thermal Cycler PERSONAL (Takara). PCR products were analysed by 1% (w/v) agarose gel electrophoresis using 1 × TAE as running buffer. DNA bands were

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Table 2. *Primers, PCR conditions and control bacterial strains/plasmids used in this study*

Name of primer	Primer sequence (5′-3′)	Target	PCR conditions			Amplicon size (bp)	Control strains	
			Denaturing	Annealing	Extension		Strain/plasmid	Ref.
tet(A)F	GCTACATCCTGCTTGCCTTC	*tet*(A)	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[a]	210	pSL18	[42]
tet(A)R	CATAGATCGCCGTGAAGAGG							
tet(B)F	TTGGTTAGGGGCAAGTTTTG	*tet*(B)	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[a]	659	pRT11	[29]
tet(B)R	GTAATGGGCCAATAACACCG							
tet(C)F	CTTGAGAGCCTTCAACCCAG	*tet*(C)	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[a]	418	pBR322	[29]
tet(C)R	ATGGTCGTCATCTACCTGCC							
tet(D)F	AAACCATTACGGCATTCTGC	*tet*(D)	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[a]	787	pSL106	[29]
tet(D)R	GACCGGATACACCATCCATC							
tet(E)F	AAACCACATCCTCCATACGC	*tet*(E)	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[a]	278	pSL1504	[43]
tet(E)R	AAATAGGCCACAACCGTCAG							
tet(G)F	GCTCGGTGGTATCTCTGCTC	*tet*(G)	98 °C, 5 s	55 °C, 15 s	72 °C, 60 s[a]	468	pJA8122	[44]
tet(G)R	AGCAACAGAATCGGGAACAC							
INT-1U	GTTCGGTCAAGGTTCTG	*intI1*	94 °C, 30 s	50 °C, 30 s	72 °C, 120 s[b]	923	*V. fluvialis*	Unpubl.
INT-1D	GCCAACTTTCAGCACATG							
5′-CS	TCGGGCATCCAAGCAGCAAGCGC	Gene cassette	98 °C, 5 s	60 °C, 15 s	72 °C, 300 s[c]	980–2800	n.a.[d]	n.a.[d]
3′-CS	TAAAAGCAGACTTGACCTGATAG							

[a] After 35 cycles, final extension step of 10 min at 72 °C was performed; [b] After 30 cycles, final extension step of 7 min at 72 °C was performed; [c] After 35 cycles, final extension step of 20 min at 72 °C was performed; [d] n.a., Not applicable.

visualized by staining the gel with ethidium bromide and viewing it under an UV transilluminator. The sizes of the PCR products were determined by using 100-bp DNA ladder and lambda *Hin*dIII digest (New England BioLabs Inc., MA, USA) as molecular size markers. PCR amplified DNAs were purified by using QIAquick PCR product purification kit (Qiagen GmbH, Hilden, Germany) and were labelled by a non-radioactive DNA labelling and detection kit (Roche Diagnostic GmbH, Mannheim, Germany). Colony hybridization test was carried out as described by Moseley et al. [17] using the Hybond-N+ nylon membrane (Amersham Biotech Corp., NJ, USA) under high-stringency conditions.

Detection of genetic determinants for tetracycline resistance

A total of 108 isolates were selected randomly from the tetracycline-resistant *E. coli* strains to detect tetracycline-resistance genetic determinants *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G) in their genomes by using the colony hybridization test as described above. If necessary, PCR was carried out using a single primer set reported by Ng et al. [18] with modified PCR conditions for confirmation of the results obtained by colony hybridization experiments. Recombinant plasmids (in *E. coli* host) containing each of the above-mentioned genes were kindly provided by Dr M. C. Roberts, University of Washington (Table 2). Each *tet* gene used as a probe was amplified by PCR with specific primer set using corresponding recombinant plasmid DNA as a template. Primer sequences and PCR conditions are described in Table 2.

Detection of class 1 integrons carrying antimicrobial resistance genes

The 108 *E. coli* strains used for detection of tetracycline-resistance genes were also screened for the presence of class 1 integrons in their genomes. Presence of integrase 1 (*intI1*) gene was examined by colony hybridization test (see below). The *intI1* gene was PCR amplified with a specific primer set (Table 2) and used as a probe. The genomic DNA of a control strain was used as templates (Table 2). The variable region, if any, of class 1 integron present in a test strain was PCR amplified to identify the drug-resistance gene by using the 5′-CS and 3′-CS primer set (Table 2). PCR amplicons obtained by this strategy

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Table 3. *Prevalence of antimicrobial resistant isolates among highly tetracycline-resistant faecal* E. coli *strains isolated from swine at an abattoir*

Herd no.	Age group[a]	No. of resistant strains against each antimicrobial agent[b] (%)					
		CHL	AMP	KAN	SXT	OFX	GEN
1 ($n=100$)	F	100 (100)	94 (94·0)	77 (77·0)	38 (38·0)	12 (12·0)	6 (6·0)
2 ($n=100$)	F	4 (4·0)	8 (8·0)	9 (9·0)	14 (14·0)	0	0
3 ($n=18$)	F	5 (27·8)	1 (5·6)	0	0	0	0
4 ($n=43$)	F	25 (58·1)	16 (37·2)	6 (14·0)	8 (18·6)	0	1 (2·3)
5 ($n=54$)	F	0	5 (9·3)	7 (13·0)	2 (3·7)	0	0
8 ($n=20$)	S	16 (80·0)	8 (40·0)	1 (5·0)	1 (5·0)	0	0
9 ($n=40$)	S	38 (95·0)	27 (67·5)	0	1 (2·5)	0	0
10 ($n=80$)	S	71 (88·8)	37 (46·3)	1 (1·3)	6 (7·5)	0	0
Total ($n=455$)		259 (56·9)	196 (43·1)	101 (22·2)	70 (15·4)	12 (2·6)	7 (1·5)
NHLR[c]		252 (97·3)	196 (100)	98 (97·0)	70 (100)	11 (91·7)	4 (57·1)

[a] F, Finisher hogs approximately 6 months old group; S, multiparous sows group.
[b] CHL, chloramphenicol; AMP, ampicillin; KAN, kanamycin; SXT, trimethoprim–sulphamethoxazole; OFX, ofloxacin; GEN, gentamicin.
[c] NHLR, Number of strains which showed four-fold resistance to the standard break-point [15].

were analysed by electrophoresis on 1·0% agarose gels. PCR-generated DNA fragments were purified from agarose gels as described above followed by sequencing in an ABI automated DNA sequencer (model 310, genetic analyser; Applied Biosystems, CA, USA) by using the same set of primers which gave the amplicon. DNA sequences were analysed by searching the GenBank database of the National Center for Biotechnology Information via the BLAST network service.

Statistical analysis

χ^2 tests and Fisher's exact test were carried out using version 8·1 of Excel (Microsoft).

RESULTS

Isolation of tetracycline-resistant *E. coli* strains

Initially, when several antimicrobial agents were used for isolation of drug-resistant *E. coli* strains from faecal samples of healthy swine from the abattoir, tetracycline-resistant strains were found to be most prevalent. Moreover, all these resistant *E. coli* strains had very high MIC values greater than 256 μg tetracycline/ml (data not shown). This striking result prompted us to examine, in more detail, the prevalence of highly tetracycline-resistant *E. coli* strains in faecal samples of healthy swine. While highly tetracycline-resistant *E. coli* strains were found to be present in 100% of sows examined it was only 61% in the case of the finisher hog group as described in Table 1. No tetracycline-resistant *E. coli* strain was isolated from the faecal samples of swine of herd nos. 6 and 7 (Table 1). Thus, a total of 455 highly tetracycline-resistant *E. coli* strains were isolated from 84 swine and the strains were subjected to further analysis.

Prevalence of multidrug-resistance phenotypes among tetracycline-resistant *E. coli* strains

When 455 tetracycline-resistant *E. coli* isolates were screened further against 11 different antimicrobial agents which are used for humans, surprisingly, several strains exhibited multidrug-resistance phenotypes (Table 3). Among these strains 259 (57%) were resistant to chloramphenicol, 196 (43%) to ampicillin, 101 (22%) to kanamycin, 70 (15%) to trimethoprim–sulphamethoxazole, 12 (3%) to ofloxacin and 7 (2%) to gentamicin (Table 3). All of the isolates which were resistant to ampicillin or trimethoprim–sulphamethoxazole were also found to be highly resistant to the above antimicrobials, and more than 90% of these strains were highly resistant to chloramphenicol, kanamycin or ofloxacin (Table 3). None of the isolates was found to be resistant to cefazolin, cefuroxime, cefotaxime, colistin and imipenem (data not shown). Interestingly, distribution of chloramphenicol, ampicillin, kanamycin,

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Table 4. *Distribution of multidrug-resistant strains among highly tetracycline-resistant faecal* E. coli *isolates*

Herd no.	Age group[a]	No. of isolates resistant to multiple antimicrobial agents (%)						
		1[b]	2[c]	3[c]	4[c]	5[c]	6[c]	7[c]
1 ($n=100$)	F	0	5 (5·0)	11 (11·0)	46 (46·0)	30 (30·0)	6 (6·0)	2 (2·0)
2 ($n=100$)	F	83 (83·0)	3 (3·0)	10 (10·0)	4 (4·0)	0	0	0
3 ($n=18$)	F	12 (66·7)	6 (33·3)	0	0	0	0	0
4 ($n=43$)	F	16 (37·2)	13 (30·3)	5 (11·6)	4 (9·3)	4 (9·3)	1 (2·3)	0
5 ($n=54$)	F	42 (77·8)	10 (18·5)	2 (3·7)	0	0	0	0
8 ($n=20$)	S	0	15 (75·0)	4 (20·0)	1 (5·0)	0	0	0
9 ($n=40$)	S	1 (2·5)	12 (30·0)	27 (67·5)	0	0	0	0
10 ($n=80$)	S	4 (5·0)	39 (48·8)	35 (43·8)	2 (2·5)	0	0	0

[a] F, Finisher hogs approximately 6 months old group; S, multiparous sows group.
[b] Tetracycline resistance.
[c] Number of antimicrobial resistant.

or trimethoprim–sulphamethoxazole-resistant strains varied considerably among the finisher hog group as described in Table 3. On the other hand, most of the strains from the multiparous sow group were resistant to chloramphenicol and more than 50% of them were resistant to ampicillin (Table 3). Details of multidrug-resistant strains distributed among highly tetracycline-resistant *E. coli* isolates obtained from faecal samples of swine are summarized in Table 4. From Table 4, it appears that the number of strains showing multiple drug resistance varied in animals from herd to herd. For example, in the finisher hog group isolates were resistant to 2–7 antimicrobials and 84% of them were resistant to more than four different drugs in herd no. 1 (Table 4). In herd no. 4, approximately 63% of the isolates were resistant to more than two antimicrobial agents varying from two to six (Table 4). However, 67–83% of strains were found to be resistant to tetracycline only in herd nos. 2, 3 and 5 (Table 4). In sow group, most of the isolates were resistant to two to three antimicrobial agents (Table 4).

Distributions of tetracycline-resistance genetic determinants and class 1 integron-associated antimicrobial resistance genes in *E. coli* strains

In order to investigate the presence of drug-resistance genes in the genomes of multidrug-resistant *E. coli* strains, we randomly selected 108 isolates in such a way that ~20% of the isolates would belong to each herd (Tables 5 and 6). The distribution of different tetracycline-resistance genetic determinants in 108 isolates was examined by colony hybridization test as described in the Materials and Methods section and the results are summarized in Table 5. Among the isolates, 95 strains carried the *tet*(B) gene in their genomes, six isolates were found positive for *tet*(A) gene, six isolates harboured both *tet*(A) and *tet*(B) genes and only one isolate showed the presence of *tet*(D) gene (Table 5). None of the isolates harboured the *tet*(C), *tet*(E) or *tet*(G) genes. To understand the contribution of class 1 integrons in multidrug resistance of 108 *E. coli* strains, the presence of the *intI1* gene in their genome was examined by colony hybridization experiment. As shown in Tables 5 and 6, 52 (48%) isolates among 108 strains examined showed the presence of the *intI1* gene in their genomes. Interestingly, almost 90% of the strains from the multiparous sow group were positive for the *intI1* gene whereas only ~30% of the strains isolated from the finisher hog group harboured the gene. This difference of percentage regarding presence of the *intI1* gene in the genomes of strains isolated from multiparous sow and finisher hog groups was statistically significant ($P<0·001$). After determining the presence of the *intI1* gene in multidrug-resistant *E. coli* strains, we examined the presence of corresponding drug-resistance gene in the strains by PCR amplification of the variable region using 5′-CS and 3′-CS primer sets as described in the Materials and Methods section. Among these strains, 24 isolates gave amplicons with four different molecular sizes (980–2800 bp) and a representative agarose gel containing all these PCR products is shown in the Figure. However, nucleotide sequence analysis of each amplicon revealed five different patterns designated Profile A to Profile E. Six strains gave a fragment of

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Table 5. *Distribution of tetracycline-resistance genetic determinants and integron-associated genes responsible for antimicrobial resistance among 108* E. coli *isolates*

Resistance phenotype[a]	No. of strains	Colony hybridization[b]				PCR product[c]	Gene cassettes[d]	Amplicon profile
		tet(A)	*tet*(B)	*tet*(D)	*intI1*			
TACOGKSxtS	2	−	+	−	+	+	*dfr17*/*aadA5*	A
TACOGSxtS	1	−	+	−	+	+	*dfr17*/*aadA5*	A
TACGKSxtS	1	−	+	−	+	+	*dhfrXII*/*orfX*/*aadA2*	B
TACOKSxt	1	−	+	−	+	+	*aacA4*/*catB3*/*dhfrI*	C
TACGKSxt	1	+	−	−	+	+	*dhfrXII*/*orfX*/*aadA2*	B
TACOSxtS	2	+	−	−	+	+	*dfr17*/*aadA5*	A
TACKSxtS	3	−	+	−	+	+	*dhfrXII*/*orfX*/*aadA2*	B
TACKSxtS	1	+	+	−	+	−		
TACKSxtS	1	−	+	−	−	−		
TACOSxt	1	−	+	−	+	+	*dfr17*/*aadA5*	A
TACKSxt	1	+	+	−	+	−		
TACKS	8	−	+	−	−	−		
TACSxtS	2	−	+	−	+	+	*dhfrXII*/*orfX*/*aadA2*	B
TACSxtS	1	+	+	−	+	+	*dhfrXII*/*orfX*/*aadA2*	B
TACK	1	−	+	−	+	−		
TACK	1	−	+	−	−	−		
TACG	1	+	−	−	−	−		
TACS	4	−	+	−	+	−		
TACS	2	−	+	−	−	−		
TACS	1	+	−	−	−	−		
TACS	1	−	+	−	+	+	*aadA*	E
TASxtS	3	−	+	−	+	+	*dhfrXII*/*orfX*/*aadA2*	B
TASxtS	1	−	+	−	+	−		
TKSxtS	1	−	+	−	+	+	*dhfrXII*/*orfX*/*aadA2*	B
TAC	3	−	+	−	+	−		
TAC	1	−	+	−	−	−		
TAS	1	−	+	−	−	−		
TKS	3	−	+	−	−	−		
TCS	4	−	+	−	+	−		
TCS	1	+	+	−	+	−		
TCS	1	−	+	−	−	−		
TA	1	−	+	−	−	−		
TC	11	−	+	−	+	−		
TC	4	−	+	−	−	−		
TC	1	+	+	−	+	−		
TS	7	−	+	−	−	−		
TS	3	−	+	−	+	+	*aadA21*	D
TS	1	+	+	−	+	+	*aadA*	E
TS	1	−	−	+	−	−		
T	22	−	+	−	−	−		
T	1	+	−	−	−	−		
Total	108	12	101	1	52	24		

[a] Tetracycline (T), ampicillin (A), chloramphenicol (C), ofloxacin (O), gentamicin (G), kanamycin (K), trimethoprim–sulphamethoxazole (Sxt), and streptomycin (S).

[b] +, Positive; −, negative.

[c] PCR was performed by using 5′-CS and 3′-CS primers (Table 2).

[d] *intI1*, integrase 1 gene; *dfr*/*dhfr*, trimethoprim resistance genes; *aadA*/*aacA*, aminoglycoside resistance genes; *orfX*, unknown open reading flame; *cat*, chloramphenicol resistance gene.

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Table 6. *Correlation of age group, presence of* intI1 *gene and class 1 integron cassette in, and profile of gene cassette among,* E. coli *strains*

Herd no.	No. of strains selected[a]	Age group[b]	No. of *intI1* +ve (%)	No. of PCR +ve[c] (%)	No. of positive strains for each gene cassette profile (%)[d]				
					A	B	C	D	E
1	(*n*=27/100)	F	13 (48·1)	11 (40·7)	6 (22·2)	4 (14·8)	1 (3·7)	0	0
2	(*n*=21/100)	F	5 (23·8)	4 (19·0)	0	1 (4·8)	0	3 (14·3)	0
3	(*n*=4/18)	F	0	0	0	0	0	0	0
4	(*n*=9/43)	F	4 (44·4)	2 (22·2)	0	2 (22·2)	0	0	0
5	(*n*=15/54)	F	2 (13·3)	2 (13·3)	0	1 (6·7)	0	0	1 (6·7)
8	(*n*=8/20)	S	6 (75·0)	1 (12·5)	0	1 (12·5)	0	0	0
9	(*n*=8/40)	S	8 (100)	1 (12·5)	0	0	0	0	1 (12·5)
10	(*n*=16/80)	S	14 (87·5)	3 (18·8)	0	3 (18·8)	0	0	0
Total	(*n*=108/455)		52 (48·1)	24 (22·2)	6 (5·6)	12 (11·1)	1 (0·9)	3 (2·8)	2 (1·9)

[a] Number of strains selected from total number of tetracycline-resistant strains isolated.
[b] F, Finisher hogs approximately 6 months old group; S, multiparous sows group.
[c] PCR was done with 5′-CS and 3′-CS primers (Table 2).
[d] For profiles see Table 5.

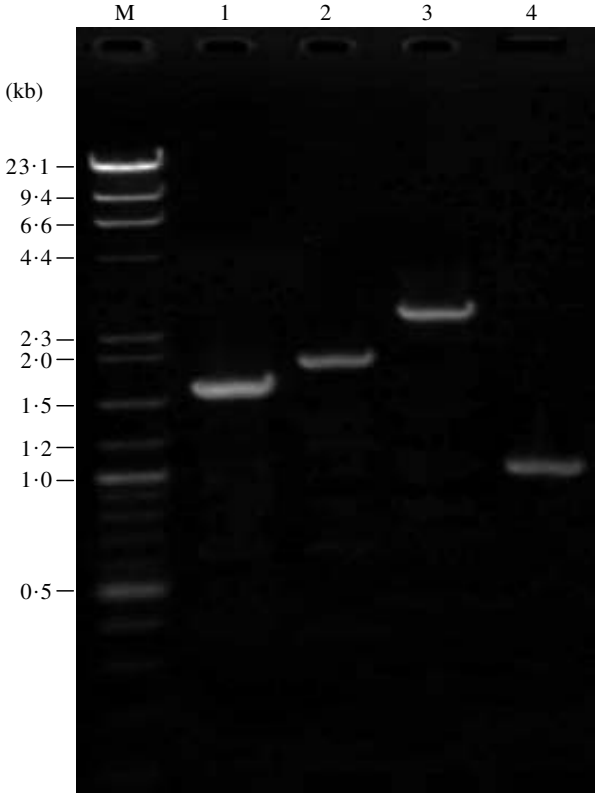

Fig. Representative PCR profiles obtained with a primer pair targeting the variable region of class 1 integrons distributed in 108 *E. coli* strains. M, 1 kb DNA ladder *Hin*dIII digest as marker: lanes 1–4 indicate PCR products of 1700, 2000, 2800 and 980 bp respectively.

1700 bp in size (Fig., lane 1) and when this amplicon was subjected to sequencing, the sequence indicated the presence of *dfr17* and *aadA5* genes (Profile A). A fragment of 2000 bp in size was most prevalent (Fig., lane 2) and was obtained from 12 strains. Sequencing of this fragment indicated the presence of *dhfrXII*, *orfX*, and *aadA2* genes (Profile B). Only one strain gave an amplicon size of 2800 bp (Fig., lane 3) and sequencing of this DNA fragment revealed the presence of *aacA4*, *catB3*, and *dhfrI* genes (Profile C). Nucleotide sequence analysis of the 980 bp fragment, which was obtained from five strains (Fig., lane 4), however, indicated the presence of two different genes. A PCR fragment of 980 bp obtained from three strains showed the presence of the *aadA21* gene (Profile D) and similar size of PCR fragment generated from two strains carried the *aadA* gene (Profile E). The function of the *orfX* gene is at present unknown [19]. The *dhfrI*, *dhfrXII* and *dfr17* genes responsible for trimethoprim resistance [19–21] were found in 19 strains. It is noteworthy that these 19 strains which were phenotypically resistant to trimethoprim were also genotypically positive for trimethoprim-resistance genes. The *aadA*, *aadA2*, *aadA5*, *aadA21* and *aacA4* genes which are responsible for aminoglycoside resistance [19–23] were found in the genomes of 24 strains. Since the aminoglycoside-resistance gene was identified in class 1 integrons present in 24 strains of *E. coli*, we examined whether these 108 strains were also phenotypically resistant to streptomycin. Determination of MIC to streptomycin correlated with antibiotic resistance genotypes of these strains. However, three strains did not correlate with streptomycin resistance although they harboured the *aadA2*, *aadA5*, or

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aacA4 gene. The *catB3* gene responsible for chloramphenicol resistance [24] was found in one strain, which was also phenotypically resistant to chloramphenicol.

DISCUSSION

In the present study, we have isolated highly tetracycline-resistant *E. coli* strains from faecal samples of healthy swine of finisher hog and multiparous sow groups, and also analysed the multidrug-resistance phenotypes of these strains. We found that in the finisher hog group there was considerable variation in isolation of tetracycline-resistant *E. coli* strains from pigs of different herds (Table 1). However, in herd nos. 1 and 2, isolation rates of resistant *E. coli* were 100% and the estimated numbers of these resistant bacteria were approximately 10^6–10^7 c.f.u./g and 10^4 c.f.u/g of faecal sample respectively. It was also estimated that the number of *E. coli* cells in normal flora of swine faeces is about 10^7 c.f.u./g of faeces [25]. It appears that in the guts of pigs of herd no. 1, almost all *E. coli* strains are highly tetracycline resistant. At present it is not clear why the number of isolation strains of tetracycline-resistant *E. coli* varied so much from one herd to another but it may be possible that such variation was caused by the level and duration of use of antimicrobials in each farm. Indeed, Mathew et al. [26] demonstrated that the patterns of antibiotic resistance in bacteria are dependent on the level of antibiotic used. Previously it was shown that extensive and long-term uses of oxytetracycline (OTC) and chlortetracycline (CTC) have apparently resulted in large populations of tetracycline-resistant bacteria in swine [26]. In this study, however, we confirmed that tetracyclines were not used for any therapeutic purposes, indicating that tetracycline-resistant strains may be evolved by use of OTC and/or CTC as metaphylaxis, prophylaxis and growth promotion or food additives in each of the farms or by unknown reason(s). On the other hand, in the multiparous sow group, the number of highly tetracycline-resistant *E. coli* strains was limited to 10^4–10^5 c.f.u. although the isolation rate was always 100% (Table 1). This result suggests that antimicrobials might be used in fewer amounts for a long period of time in adult pigs in comparison to certain young pigs.

Apart from tetracycline resistance we also investigated the multidrug-resistance phenotypes of 455 *E. coli* strains isolated in this study against 11 different antimicrobials (Table 3). It is interesting to note that diversity of prevalence of multidrug-resistant *E. coli* was observed especially among the finisher hog groups (Table 4). The results suggest that antimicrobials might have been used in large quantities for a short period of time in certain finisher hog groups, rather than multiparous sow groups, to protect the young animals from infectious diseases and to promote them in gaining weight in order to increase production efficiency and simultaneously reduce economical loss.

In this study, the randomly selected 108 tetracycline-resistant *E. coli* isolates were found to be positive for the *tet*(A), *tet*(B) or *tet*(D) gene, or *tet*(A) and *tet*(B) genes (Table 5). The products of these genes are involved in active efflux of the antibiotic from cell in Gram-negative bacteria [2, 3, 7, 9, 27]. Previous reports indicate that the tetracycline-resistance genetic determinant *tet*(B) is most common and widespread among enteric bacteria [8, 28, 29]. In the present study, the *tet*(B) gene was also found to be the most common among highly tetracycline-resistant *E. coli* strains (Table 5) and thus, our result is highly consistent with the results obtained from previous studies [8, 28, 29].

Implications of class 1 integron, which play a role in the horizontal transfer of genes in bacteria [30–33], in multidrug resistance have been reported by several groups [34–36] and this study further supports this observation. Interestingly, the *intI1* gene was found to be more prevalent in multiparous sow than in finisher hog groups. However, the presence of drug-resistance genes amplified by 5′-CS and 3′-CS was not correlated with the frequency of the presence of the *intI1* gene in the sow group. Surprisingly, no class 1 integron-positive *E. coli* strains were isolated from animals in herd no. 3 (Table 6). From the animals of this herd only 18 highly tetracycline-resistant *E. coli* strains were isolated and these strains were found to be resistant to only one or two antimicrobial agents (Tables 1 and 4). In contrast, class 1 integron-positive *E. coli* strains were most prevalent in the intestinal tracts of animals of herd nos. 1 and 4 and these strains were also found to be multidrug resistant with two to more than seven antimicrobials indicating that young pigs might have been exposed to a number of antimicrobials within very short period of time compared to adult pigs. Thus, in the genomes of *E. coli* strains isolated from adult pigs, the class 1 integron cassette could be detected but

it was devoid of a particular drug-resistance gene, possibly because of less use of antimicrobials. On the other hand, *E. coli* strains isolated from young pigs were most probably able to acquire the drug-resistance genes in their class 1 integrons very efficiently because of extensive exposure to antimicrobials in this age group within a very short time. However, further investigation is needed to clarify this point but our data clearly indicate that the class 1 integron indeed plays a role in the genesis of multidrug-resistant *E. coli* strains.

In addition, our data indicated that it is also possible to produce healthy animals, which do not harbour multidrug-resistant strains. It has been reported earlier that tetracycline-resistant faecal coliforms of swine decreased from 82% to 42% after the withdrawal of the drug in a herd for 126 months [37]. Indeed, in the case of animals of herd no. 5, all animals had highly tetracycline-resistant *E. coli* strains, however, their isolation rate was 54%, indicating that sufficient withdrawal period may have contributed to reduce the rate and number of resistant *E. coli* strains in their faeces before slaughtering. It has been reported that any form of antimicrobial exposure will increase the prevalence of antimicrobial resistance and multiple drug resistance in faecal bacteria [38]. It has also been described that non-pathogenic *E. coli* from swine may represent a potential reservoir of antibiotic-resistance genes that may be transferred to pathogenic organisms [36, 39] and, thus, may convert them to multidrug resistant. From 2000 the Danish government has undertaken an unprecedented challenge by banning the use of antibiotics for pigs as a growth-promoting factor [40]. However, they later found that mortality and infectious diseases increased among animals, resulting in a rather enhanced use of antibiotics for therapeutics, and economic loss [40]. There is another report mentioning that the consumers and the food-producing industry presumably receive economic benefits from the use of antimicrobials in animal feed [41]. Therefore, it cannot be easily concluded that the use of antibiotics for growth promotion is always incorrect. Nevertheless, it may be useful for metaphylaxis, prophylaxis or growth promotion if antimicrobials are used under proper control in certain levels but if the dose of the drug is increased, or its duration of use is too long, then the number of drug-resistant bacterial strains including multidrug resistance may increase and will cause major threats to public health.

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