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Characterization of chloramphenicol acetyltransferase gene by multiplex polymerase chain reaction in multidrug-resistant strains isolated from aquatic environments

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Abstract

Multidrug-resistant isolates carrying the chloramphenicol resistance gene were obtained from aquatic farms in various locations in Korea and studied to determine the distribution and origin of the *cat* gene. Out of the 134 isolates examined, 10 showed multidrug resistance to several different antibiotics including chloramphenicol (CP). One of these 10 multidrug-resistant bacteria, *Vibrio damsela* JE1 (*V. damsela* JE1) contained a transferable R plasmid encoding CP and tetracycline (TC) resistance genes. The presence of the R plasmid was confirmed by conjugation using the chromocult medium (CC) as a selective and differential medium for transconjugants with identification based on the growth or colors of the colonies. To determine the types of chloramphenicol acetyltransferase genes (*cat*), polymerase chain reaction (PCR) with primers derived from the variable and conserved regions of different types *cat* genes, appeared to be a very specific and sensitive method. Additionally, we developed the multiplex PCR that allowed us to determine the types of *cat* genes by the different sizes of the resulting PCR products in a single reaction tube. With the PCR method, we determined that all multidrug-resistant isolates of *Vibrio* from the farms of the South and East Sea that contained the *cat* gene carried type IV gene in an R plasmid or other nucleic acids, and the remaining isolates including *Edwardsiella tarda*, *Aeromonas hydrophila* and *Shewanella* sp. carried the type II gene. This result suggested that the type of *cat* gene in the multidrug-resistant bacteria

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originated from a very limited environmental or biological source and was not dependent on the location of the area of isolation in Korea.

Keywords: Aquatic farm; Multidrug resistance; Chromocult medium; Conjugation; Chloramphenicol acetyltransferase gene; Multiplex PCR

1. Introduction

Epidemiological surveillance of drug-resistant strains of fish pathogens has been undertaken to determine the origins and prevalence of multidrug resistance that is related or unrelated to the presence of R plasmids, and to find a way to prevent the spread of these drug-resistant strains in fish farms. Moreover, similar resistance patterns and the presence of selected resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* isolated from humans, broilers, and pigs in Denmark (Aarestrup et al., 2000) indicated that transmission of resistant bacteria or resistance genes takes place between humans and animals.

By permission of the Food and Drug Administration (FDA) to use tetracycline (TC) for humans and aquatic animals, most of the genetic studies were focused on the tetracycline resistance gene (*tet*) in comparison with other antibiotic resistance genes in fisheries sciences (Guardabassi et al., 2000; Rhodes et al., 2000; Anderson and Sandaa, 1994; Levy, 1989). With TC, chloramphenicol (CP) is also an effective therapeutic agent that has been widely used in fish farms. However, its use has been prohibited in Korea and Japan since 1983. Thiamphenicol and florfenicol, derivatives of CP, have also been used to treat bacterial infectious disease in fish farms (Samuelsen et al., 1998; Ho et al., 2000); however, CP-resistant strains of fish-pathogenic bacteria have remained prevalent (Kim and Aoki, 1993; Arcangioli et al., 2000). Thus, *cat* genes derived from limited sources are being transferred in the aquatic microorganisms without a high selective environmental pressure. In terms of this, analysis of the distribution and DNA structure of the *cat* genes in multidrug-resistant fish pathogens would be worthwhile in order to understand the origin of resistance genes and their relationships to human or animal pathogens.

Chloramphenicol resistance is due to inactivation of the drug mediated by chloramphenicol acetyltransferases (CAT) that have a trimeric structure composed of identical subunits (Leslie, 1990; Schwarz and Cardoso, 1991; Shaw, 1983). There are several types of *cat* genes with different nucleic acid sequences, and the resulting CAT enzymes have different structures (Roberts et al., 1982). Zaidenzaig and Shaw (1976), using enzymologic, biochemical and immunological methods, have classified the CATs produced by the R plasmid in Gram-negative bacteria into enzyme types I, II, and III.

The CAT of R plasmids from *Vibrio anguillarum* have been classified into two types (Masuyoshi et al., 1988; Aoki, 1988). One type, from an R plasmid first detected in 1980, was classified as CAT II, but the other type, detected before 1977, could not be classified as either CAT I, II, or III and therefore has been designated as a new type, CAT IV (Zhao and Aoki, 1992; Aoki, 1988). The *cat* or other resistance genes have been analyzed by molecular biological methods including PCR (Arcangioli et al., 2000). However, multiplex

PCR methods have not been developed to detect and discriminate the four different types of *cat* in the isolates of fish pathogens in a single reaction tube.

The objective of this study was to apply differential media for the isolation of transconjugants in conjugation assays for the transfer of R plasmids and to define the prevalence of the types of *cat* genes by developing multiplex PCR methods for use with clinical isolates of fish pathogens.

2. Materials and methods

2.1. Bacterial cultures

A total of 134 clinical isolates of fish-pathogenic bacterial agents isolated from 1993 to 1999 from different areas of Korea were evaluated. The control laboratory positive strains used in this study were kindly provided by Dr. E. Kim and are listed in Table 1 along with their respective characteristics (Kim and Aoki, 1993; Aoki et al.. 1985). All reference *Escherichia coli* (*E. coli*) strains and the clinical isolates in this laboratory were grown at 37 and 25 °C in tryptose soy broth (TSB) (Difco, Detroit, MI, USA), respectively, and were stored in 50% glycerol at −72 °C until use.

2.2. Drug sensitivity tests

All of the clinical isolates grown aerobically in tryptose soy broth (TSB) supplemented with 1% (w/v) NaCl at 25 °C for 18 h were diluted in phosphate-buffered saline (PBS) (10^5 colony forming units/25 μl) and dropped on plates of the selective media tryptose soy agar (TSA) containing 10 μg ml^{-1} CP (Sigma, USA). CP resistant isolates that showed more than 10 colonies on the selective media were further analyzed to determine the presence of other resistances on a TSA plate containing 30 μg tetracycline (TC), 2 μg oxolinic acid (OA), 30 μg nalidixic acid, 10 μg ampicillin (AMP), 10 μg colistin (CL), 30 μg kanamycin (KM) and 10 μg streptomycin (SM). Multidrug-resistant isolates were identified by an API 20E kit (BioMerieux, Marcy, France) and by conventional laboratory methods including Gram stain, growth on selective media, and colony morphology. Minimum inhibitory concentrations (MIC) of antibiotics for transconjugants were determined by the TSA plate doubling dilution method with incubation at 37 and 25 °C for *E. coli* strains and laboratory isolates, respectively, for 24 h.

Table 1
Reference strains used in this study

E. coli strains	Resistance marker	Genetic characteristics (type of *cat* gene)
K-12 HB101	SM^r	Recipient cell
C-600 pJAPE 8232	TC^r, CP^r, KM^r, SA^r, SM^r	R plasmid (type I)
C-600 W3102	CP^r, SM^r	Plasmid (type II)
C-600 R387	CP^r, SM^r	Plasmid (type III)
C-600 pJA 8122	CP^r, TC^r, OA^r, SM^r	R plasmid (type IV)

SM, streptomycin; TC, tetracycline; CP, chloramphenicol; KM, kanamycin; SA, sulfonamide; OA, oxolinic acid.

2.3. Conjugal transfer assay

Transferability of resistance markers was examined by using filter mating and chromocult coliform (CC) agar plates (Merck, Darmstadt, Germany) as a selective and differential medium. Donor and recipient cells were grown overnight in 4 ml of TSB. The donor cell culture (0.2 ml) was added to 1.8 ml of the recipient cell culture and mixed. The mixture was passed through a nitrocellulose 0.45 μm filter (Millipore, Bedford, MA, USA.). The filter was placed with the bacterial cells touching the surface of a TSA plate. After incubation at 30 °C for 24 h, the filter was removed and mixed with 10 ml of TSB. The mating mixture was spread onto CC agar plates containing CP. After incubation at 30 °C for 24 h, the colors of the colonies were observed.

2.4. Preparation of R plasmid DNA

Plasmid DNA was extracted using a modification of the method of Kado and Liu (1981). Isolates were grown in brain heart infusion (BHI) broth (Difco) at 30 °C for 6 h on a shaker incubator. Cultures (25 ml) were centrifuged at $6000 \times g$ for 15 min. The supernatant was discarded and the pellet was resuspended in 1 ml, Tris-acetate/EDTA, pH 8.0 buffer. An aliquot (2 ml) of lysing solution (3% sodium dodecyl sulfate , 50 mM Tris, pH 12.6) was added to the cell suspension, and the samples were incubated for 60 min at 55 °C. A 6-ml quantity of phenol–chloroform (1:1) (Sigma) was mixed, and the samples were centrifuged. The supernatant was taken, and was analysed without purification, by electrophoresis on 0.7% SEAKEM Gold agarose (FMC Bio Products) gels containing 0.5 μg ml^{-1} ethidium bromide, using a potential of 80 V for 1.5–2 h.

2.5. DNA extraction

All of the bacterial species were grown aerobically on TSB supplemented with 1% (w/v) NaCl at 25 °C for 18 h. Cultured cells were harvested by centrifugation at $8000 \times g$ for 10 min and lysed with 5.5% sodium dodesyl sulfate/ 0.125 mg ml^{-1} proteinase K (BM, Germany) solution. Bacterial nucleic acids were extracted by a phenol–chloroform–isoamyl alcohol (25:24:1 v/v/v) mixture and chloroform–isoamyl alcohol (24:1 v/v) mixture. The nucleic acids were precipitated by adding two volumes of ethanol in the presence of 0.3 M sodium acetate.

2.6. Primer design and PCR

Four sense primers were designed: C-1, C-2, C-3, and C-4, which were complementary to the specific regions of the *cat I*, *cat II*, *cat III,* and *cat IV* genes, respectively. One antisense primer, C-R was derived from the conserved nucleotide sequence of the four different types of *cat* gene sequences (Table 2). These primers were designed with the aid of gene alignment using the MACAW (Version 2.0.5. National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA) program for four different types of *cat* gene sequences retrieved from the Entrez database (Accession numbers M16323, X53796, X07848, S48276, respectively). All primers were synthesized with an

Table 2
Primers used in PCR

Primer	Orientation	Positions	Base sequence
C-1	Sense	*cat I*, 245–264	5′-GGTGATATGGGATAGTGTT-3′
C-2	Sense	*cat II*, 15–35	5′-GATTGACCTGAATACCTGGAA-3′
C-3	Sense	*cat III*, 307–326	5′-CCATACTCATCCGATATTGA-3′
C-4	Sense	*cat IV*, 122–141	5′-CCGGTAAAGCGAAATTGTAT-3′
C-R	Antisense	*cat II*, 581–562[a]	5′-CCATCACATACTGCATGATG-3′

[a] Sequence positions are given according to the *E. coli* K-12 (accession number; X53796) numbering system.

automated DNA synthesizer (Bioneer, Taejon, Korea) by the phosphoramidite method. Multiplex PCR amplification was carried out in a 50-μl reaction mixture containing the extracted bacterial nucleic acid (100 ng of total nucleic acid isolated from one or two different bacteria), 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 0.5% Tween-20, 200 μM each dNTP, 1 μM each antisense and four sense primer, 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) with an Perkin-Elmer 2400 thermal cycler (Perkin-Elmer). Amplification consisted of 30 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min in 0.2 ml thin-walled tubes. The results of amplification were analyzed by 1.5% agarose gel electrophoresis. The PCR products were purified by agarose gel electrophoresis using a Prep-A-Gene DNA Purification systems (Bio-Rad) and sequenced using a Big dye terminator cycle DNA sequencing kit (ABI PRISM, PE Applied Biosystems, Foster City, CA, USA) and an automatic sequencer.

3. Results

3.1. Isolation of the multidrug-resistant fish-pathogenic bacteria

Ten CP resistant isolates of different fish-pathogenic bacteria were obtained using the CP (10 μg ml^{-1}) containing TSA plate as a selective medium from 134 epizootics of cultured fish including flounder and eel . The analysis of the resistance patterns of the isolates was extended to KM, AMP, CL, NA and OA, and identified that all 10 CP-resistant isolates were multidrug-resistant bacteria (data not shown). To determine the involvement of transferable R plasmids encoding CAT, the transfer of the *cat* gene to the recipient, *E. coli* K-12 HB101, was analyzed by conjugation experiments on a CC agar plate containing CP. After conjugation, it was easy to distinguish the transconjugant cells that have the same types of antibiotic resistance from those of the donor on the CC agar plate by the growth or observation of the colony colors (Table 3). With this method, 1 out of 10 multidrug-resistant isolates, *Vibrio damsela* JE 1 (*V. damsela* JE 1), was found to contain a transferable plasmid encoding CAT. Gel electrophoresis (Fig. 1) demonstrated the presence of the R plasmid conferring resistance in the *V. damsela* JE 1 isolate and subsequently in the *E. coli* K-12 HB101 (pVD) transconjugants. The plasmids isolated from the donor, *V. damsela* JE 1, and transconjugant, *E. coli* K-12 HB101 (pVD), both appear to be more than 200 kb pairs in length assumed by the comparison with the R plasmid of 200 kb in *E. coli* pJAPE 8232 (Kim and Aoki, 1993). Although the *V. damsela*

Table 3
Various colony colors of different strains of bacteria on CC agar plate

Strains	Colors of colony
E. coli K-12 HB101	Dark-blue to violet
E. coli C-600 pJAPE 8232	Light-blue
E. tarda	Colorless
Vibrio sp.	No growth
Aeromonas sp.	Purple
Shewanella sp.	No growth

JE 1 contained another additional small plasmid, its function and transfer activity were not analyzed.

The result obtained from the determination of the MIC was that the transconjugant, *E. coli* HB101 (pVD), had a different antibiotic resistance pattern from that of the donor *V. damsela* JE 1 (Table 4). The pattern, which showed only resistance to TC and CP but not to KM and AMP in transconjugant *E. coli* K-12 HB101 (pVD), suggested the different location of these resistance genes, i.e., that the former were located in transferable nucleic acids and the latter were located in non-transferable nucleic acids in donor cells.

3.2. Distribution of cat genes among multidrug-resistant isolates

To define the characteristics of *cat* in multidrug-resistant isolates of fish pathogens from Korea, multiplex PCR with the primers against the *cat I*, *II*, *III*, *IV* genes was developed (Table 2). Multiplex PCR with the mixture of a single antisense primer and four sense primers derived from the conserved region and the different variable regions of the *cat I*, *II*, *III*, *IV* genes, respectively, were specific for the detection of each type of *cat* gene and

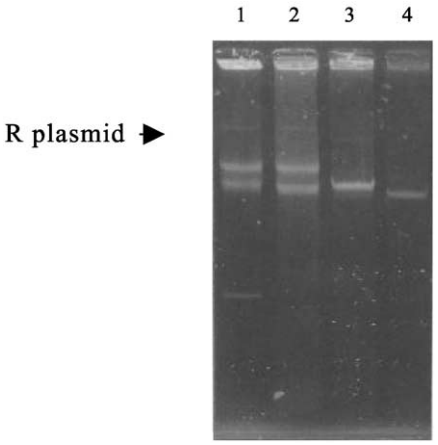

Fig. 1. Agarose gel electrophoresis of R plasmids. Lane 1, *V. damsela* JE1; Lane 2, *E. coli* K-12 HB101 (pVD); Lane 3, *E. coli* pJAPE 8232; Lane 4, *E. coli* K-12 HB101.

Table 4
MIC of antibiotics for *V. damsela* JE1 and its transconjugant

Bacteria	Minimum inhibitory concentration (μg/ml)						
	CP	TC	OA	NA	AMP	CL	KM
E. coli K-12 HB101	12.5	1.56	0.312	6.25	6.25	0.39	6.25
V. damsela JE1	>100	>100	1.25	12.5	>100	12.5	>100
E. coli K-12 HB101(pVD)[a]	>100	>100	0.312	6.25	12.5	1.56	6.25

[a] *E. coli* K-12 HB101 was used as a recipient of R plasmid from *V. damsela* JE1.

able to discriminate the *cat I*, *II*, *III*, and *IV* genes by the different sizes of the products: 349, 567, 275, 451 bp, respectively (Fig. 2). It also was powerful enough to detect more than two types of *cat* gene present in the reaction mixture formed by the addition of different nucleic acid extracted from each different bacterium carrying a specific type of *cat* gene (Fig. 2).

Using multiplex PCR, the distribution of different types of *cat* in 10 multidrug-resistant isolates from Korea was analyzed (Fig. 3). Interestingly, we found that all the isolates of *Vibrio* species (*V. damsela* JE 1, *Vibrio* sp. KS 6 and JE 5) carried the *cat IV* gene. In contrast, all other species including five isolates of *Edwardsiella tarda* (KS 1, KS 2, JH 10, RE 1, RE 23), *Aeromonas hydrophila* H 1 and *Shewanella* sp. harbored the *cat II* gene (Fig. 3). Sequenced PCR products of the *cat II* and *IV* genes appeared to be 100%

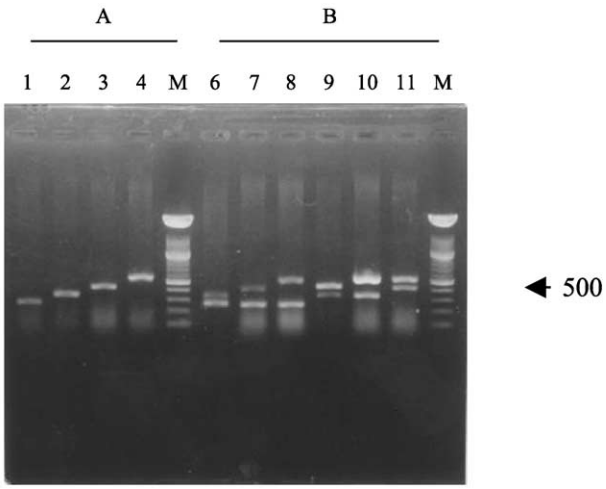

Fig. 2. Specificity of multiplex PCR with primers designed for analysis of different *cat* genes. Used templates for *cat I* (349bp), *cat II* (567bp), *cat III* (275bp), and *cat IV* (451bp) were the total nucleic acids isolated from *E. coli* pJAPE 8232, *E. coli* C600 W3102, *E. coli* C600 R387 and *E. coli* pJA 8122, respectively. Product of multiplex PCR gene amplification with: (A) a single *cat* gene template; (B) a mixture of two different *cat* gene templates; Lane 1, *cat III*; Lane 2, *cat I*; Lane 3, *cat IV*; Lane 4, *cat II* ; Lane 6, *cat III* and *cat I*; Lane 7, *cat III* and *cat IV*; Lane 8, *cat III* and *cat II*; Lane 9, *cat I* and *cat IV*; Lane 10, *cat I* and *cat II*; Lane 11, *cat IV* and *cat II*; Lane M, 100 bp DNA ladder as a marker.

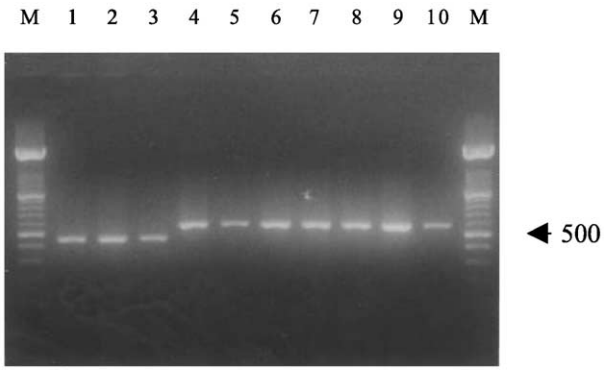

Fig. 3. Electrophoretic analysis of PCR products obtained by the amplification of *cat* genes in the clinical isolates with multi-primers of C-1, C-2, C-3, C-4 and C-R. Lane 1, *Vibrio* sp. KS 6; Lane 2, *Vibrio* sp. JE 5; Lane 3, *V. damsela* JE 1; Lane 4, *Shewanella* sp.; Lane 5, *A. hydrophila* H 1; Lane 6, *E. tarda* KS 2; Lane 7, *E. tarda* KS 1; Lane 8, *E. tarda* JH 10; Lane 9, *E. tarda* RE 23; Lane 10, *E. tarda* RE 1; Lane M, 100 bp DNA ladder as a marker.

homologous with the reported ones (accession numbers X53796 and S48276). Other types (*cat I* and *III* genes) or the presence of more than two different types of *cat* gene were not found in any CP-resistant fish-pathogenic isolates used in this experiment.

4. Discussion

In the present study, of 134 Korean isolates of fish-pathogenic bacteria, there appeared to be 10 isolates resistant to CP and various chemotherapeutic agents, and a transferable R plasmid was detected in only one (Fig. 1). It would not be easy to compare the frequency of multidrug-resistant isolates with those of other studies since we were interested in the isolates that carried *cat* together with other resistance. Thus, there would be some strains resistant to single or combined drugs, other than CP, among the 134 isolates. Additionally, it is possible that the actual number of isolates carrying R plasmids may have been higher since the failure to show transfer does not prove the absence of the plasmid. In addition, resistant isolates other than the 10 that we isolated and analyzed may also have had transferable R plasmids missing the *cat* gene.

In the R plasmid transfer analysis, it is sometimes difficult to identify a drug that would discriminate the transconjugants from the donor cells on selective media. This may limit the range of selection for a usable recipient in conjugation experiments for R plasmid containing multidrug-resistant genes. Although MacConkey's agar has been used to overcome this problem as a selective medium, it is not powerful enough to select or discriminate many different Enterobacterial microorganisms (Aurora et al., 1992). In the present study, all donor cells, isolates, have resistance against streptomycin, which is the only type of drug resistance in the recipient, *E. coli* K-12 HB101. Thus, it was necessary to develop a selective method for transconjugant isolation from the mixture of donor and recipient cells in the conjugation experiment.

On the CC agar, designed for the morphological differentiation (or selection) of fecal enterococci and a high level of discrimination efficacy (Turner et al., 2000; Byamukama et al., 2000; Ogden et al., 1998), many bacteria related to *Enterobacteriaceae* showed various colony colors. On the CC media containing CP, it was very efficient to isolate the transconjugant from different donor cells that showed various colony colors or no growth (*Vibrio* sp.) on CC medium itself in this experiment (Table 3). However, it should be noted that this method is limited for use with recipient cells that cannot grow or have the same colony colors as those of donor cells grown on CC agar.

For further characterization of the *cat* genes, we designed four sense primers against variable regions and one antisense primer against the conserved region of different *cat* genes and developed a multiplex PCR for the detection and discrimination of each specific type of *cat* gene carried in the fish-pathogenic isolates (Fig. 2). It was found that multiplex PCR with four sense primers and one antisense primer in a single reaction tube allowed us to discriminate the type of *cat* gene depending upon the different sizes of the amplified products. Additionally, it was powerful enough to detect and discriminate all the different combinations of two different types of *cat* gene. However, in some cases for more than three different types of *cat* gene, depend upon the combination, one of the *cat* genes, especially type IV, did not produce the corresponding amplified DNA fragments in this multiplex PCR system (data not shown). This might be derived from the unbalanced binding activity of each different sense primer to the corresponding templates or different copy numbers of plasmid in a given weight of DNA.

Although multiplex PCR has been applied to detect multidrug-resistant bacteria in previous studies, the methods required the presence of each different set of primers against all different target genes (Katsuno et al., 2001; Khan et al., 2000). There has not been a report of the use of multiplex PCR to detect and discriminate four different types of *cat* gene carried in microorganisms, especially with a single antisense primer and four sense primers. In the analysis of *cat* genes by multiplex PCR in the 10 multidrug-resistant isolates, three *Vibrio* species and the remaining isolates including five *E. tarda*, one *A. hydrophila* and *Shewanella* sp. carried the *cat IV* and *cat II* gene, respectively (Fig. 3). Additionally, no isolates used in this study were carrying more than two different types of *cat* gene. This was different from the studies of the *tet* gene which showed the presence of two or more different types of *tet* gene in a given drug-resistant bacterium (Pang et al., 1994; Anderson and Sandaa, 1994; Warsa et al., 1996). Thus, it might be said that multidrug-resistant fish pathogens isolated in Korea carry restricted types of *cat* gene, only *cat II* or *cat IV*, depending upon the species of microorganism. However, the prevalence of the *cat II* gene in different fish pathogens from Korea is different from that found by Shaw (1983), who reported the widespread distribution of *cat I* and *cat III* genes and a less common incidence of the *cat II* gene in different Gram-negative and Gram-positive bacteria. Thus, this question should be analyzed further with larger numbers of bacteria isolated from different environments, geographical locations and at different times. Moreover, the *cat IV* gene that was found in Japan from the fish pathogens isolated before 1977 (Aoki, 1988), and which is prevalent in the *Vibrio* species of Korea at the present time, could yield very interesting information allowing speculation about the origin and spread of drug resistance genes between two neighboring countries.

Other comparative studies should be done to demonstrate the origin and evolution of the drug-resistance genes derived from the pathogens of humans, domestic animals, and fish.

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References

Aarestrup, F.M., Agerso, Y., Gerner-Smidt, P., Madsen, M., Jensen, L.B., 2000. Comparison of antimicrobial phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn. Microbiol. Infect. Dis. 37, 127–137.

Anderson, S.R., Sandaa, R.A., 1994. Distribution of tetracycline resistance determinants among gram-negative bacteria isolated from polluted and unpolluted sediments. Appl. Environ. Microbiol. 60, 908–912.

Aoki, T., 1988. Drug-resistant plasmids from fish pathogens. Microbiol. Sci. 5, 219–223.

Aoki, T., Kanazawa, T., Kitao, T., 1985. Epidemiological surveillance of drug resistant *Vibrio anguillarum* strains. Fish Pathol. 20, 199–208.

Arcangioli, M.A., Leroy-Setrin, S., Maetel, J.L., Chaslus-Dancla, E., 2000. Evolution of chloramphenicol resistance, with emergence of cross-resistance to florfenicol, in bovine *Salmonella typhimurium* strains implicates definitive phage type (DT)104. J. Med. Microbiol. 49, 103–110.

Aurora, F., Muela, A.A., Cisterna, R., Iriberri, J., Barcina, I., 1992. Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. Appl. Environ. Microbiol. 58, 392–398.

Byamukama, D., Kansiime, F., Mach, R.L., Farnleitner, A.H., 2000. Determination of *Escherichia coli* contamination with chromocult coliform agar showed a high level of discrimination efficiency for differing fecal pollution levels in tropical waters of Kampala, Uganda. Appl. Environ. Microbiol. 66, 864–868.

Guardabassi, L., Dijkshoorn, L., Collard, J.M., Olsen, J.E., Dalsgaard, A., 2000. Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter strains*. J. Med. Microbiol. 49, 929–936.

Ho, S.P., Hsu, T.Y., Wang, W.S., 2000. Antibacterial effect of chloramphenicol, thiamphenicol and florfenicol against aquatic animal bacteria. J. Vet. Med. Sci. 62, 479–485.

Kado, C.I., Liu, S.T., 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145, 1365–1373.

Katsuno, S., Takashi, M., Ohshima, S., Ohta, M., Kato, N., Kurokawa, H., Arakawa, Y., 2001. Direct screening of the IMP-1 metallo-beta-lactamase gene (blaIMP) from urine samples by polymerase chain reaction. Int. J. Urol. 8, 110–117.

Khan, A.A., Nawax, M.S., Khan, S.A., Cerniglia, C.E., 2000. Detection of multidrug resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. FEMS Microbiol. Lett. 182, 355–360.

Kim, E., Aoki, T., 1993. The structure of the chloramphenicol resistance gene on a transferable R plasmid from the fish pathogen, *Pasteurella piscicida*. Microbiol. Immunol. 37, 705–712.

Leslie, A.G.W., 1990. Refined crystal structure of type III chloramphenicol acetyltransferase at 1.75Å resolution. J. Mol. Biol. 213, 167–186.

Levy, S.B., 1989. Evolution and spread of tetracycline resistance determinants. J. Antimicrob. Chemother. 24, 1–3.

Masuyoshi, S., Okubo, T., Inoue, M., Mitsuhashi, S., 1988. Purification and some properties of a chloramphenicol acetyltransferase mediated by plasmid from *Vibrio anguillarum*. J. Biochem. 104, 130–135.

Ogden, I.D., Brown, G.C., Gallacher, S., Garthwaite, P.H., Gennari, M., Gonzalez, M.P., Jorgensen, L.B.,

Lunestad, B.T., MacRae, M., Nunes, M.C., Petersen, A.C., Rosnes, J.T., Vliegenthart, J., 1998. An interlaboratory study to find an alternative to the MPN technique for enumerating *Escherichia coli* in shellfish. Int. J. Food Microbiol. 40, 57–64.

Pang, Y., Bosch, T., Roberts, M.C., 1994. Single polymerase chain reaction for detection of tetracycline-resistant Tet K and Tet L. Mol. Cell. Probes 8, 417–422.

Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Smith, P., Pickup, R.W., 2000. Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: implication of Tn1721 in dissemination of the tetracycline resistance determinant *tet* A. Appl. Environ. Microbiol. 66, 3883–3890.

Roberts, M., Corney, A., Shaw, W.V., 1982. Molecular characterization of three chloramphenicol acetyltransferases isolated from *Hemophilus influenzae*. J. Bacteriol. 151, 737–741.

Samuelsen, O.B., Hjeltnes, B., Glette, J., 1998. Efficacy of orally administered florfenicol in the treatment of furunculosis in Atlantic salmon. J. Aquat. Anim. Health 10, 55–61.

Schwarz, S., Cardoso, M., 1991. Nucleotide sequence and phylogeny of a chloramphenicol acetyltransferase encoded by the plasmid pSCS7 from *Staphylococcus aureus*. Antimicrob. Agents Chemother. 35, 1551–1556.

Shaw, W.V., 1983. Chloramphenicol acetyltransferase: enzymology and molecular biology. Crit. Rev. Biochem. 14, 1–46.

Turner, K.M., Restaino, L., Frampton, E.W., 2000. Efficacy of chromocult coliform agar for coliform and *Escherichia coil* detection in foods. J. Food Prot. 63, 539–541.

Warsa, U.C., Nonoyama, M., Ida, T., Okamoto, T., Okubo, T., Shimauchi, C., Kuga, A., Inoue, M., 1996. Detection of tet(K) and tet(M) in *Staphylococcus aureus* of Asian countries by polymerase chain reaction. J. Antibiot. 49, 1127–1132.

Zaidenzaig, Y., Shaw, W.V., 1976. Affinity and hydrophobic chromatography of three variants of chloramphenicol acetyltransferase specified by R factors in *Escherichia coli*. FEBS Lett. 62, 266–271.

Zhao, J., Aoki, T., 1992. Cloning and nucleotide sequence analysis of a chloramphenicol acetyltransferase gene from *Vibrio anguillarum*. Microbiol. Immunol. 36, 695–705.