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Detection of beta-lactam antibiotic resistant genes in *Escherichia coli* from porcine fecal samples using DNA chip

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Abstract

This study was conducted to detect β-lactam antibiotic-resistant genes in the 400 *E coli* isolates from porcine fecal samples in Korea by a DNA chip. The DNA chip contains the specific probe DNAs of the β-lactam antibiotic-resistant genes that had been labeled with a mixture of primer set designed to amplify specific genes (*PSE, OXA, FOX, MEN, CMY, TEM, SHV, OXY* and *AmpC*) using a multiplex polymerase chain reaction (PCR). Of 400 isolates 339 contained at least one β-lactamases gene. Resistance to β-lactamases was mediated mainly by *AmpC* (n = 339, 100%), and followed by *TEM* (n = 200, 59.0%), *CMY* (n = 101, 29.8%), *PSE* (n = 30, 8.9%) and both *OXA* and *SHV* genes (n = 20, 5.9%), while the *FOX*, *MEN* and *OXY* genes were not detected. The other sixty-one did not contain any β-lactamase genes even though they were resistant to antimicrobial drugs. In conclusion, the DNA chip system can be used as a rapid and reliable method for detecting of β-lactamases genes, which will help veterinarians select the antibiotics for monitoring and treating of animal diseases.

Key words : Beta-lactam, *Escherichia coli*, DNA chip, Pig

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Introduction

Antibiotic-resistant *Enterobacteriaceae* cause major clinical problems in both animal and human healthcare. These problems are related to the increasing of antimicrobial consumption[1]. Overuse of antibiotics in both medicine and agriculture-agrifood industry is main factors[2].

Beta-lactamase resistant *Escherichia*

coli has high incidence of resistance to extended-spectrum-lactam (ESBL) among pathogens in Korea[2]. The prevalence of β-lactamases bacteria in animals has been increased since 1990s[3]. Generally, β-lactamases can be classified into four main groups and eight subgroups according to their functional and structural characteristics[4]. Resistance to β-lactam antimicrobial agents in *E coli* is primarily mediated by β-lactamases, which hydrolyze the β-lactam ring and thus inactivate the antibiotics[5]. Many different β-lactamases have been described[3,5].

The classical *TEM*-1, *TEM*-2, and *SHV*-1 enzymes are the predominant plasmid-mediated β-lactamases of Gram-negative rods. Six different nucleotide sequences, called bla*TEM*-1a, bla*TEM*-1b, bla*TEM*-bla*TEM*-1d, bla*TEM*-1e, and bla-*TEM*-1f, have been described to codify the same *TEM*-1 β-lactamase[6]. Some variants of the *TEM*-*TEM*-2, and *SHV*-1 β-lactamases have emerged as a result of single amino acid substitutions in the sequences of the genes which render the extended-spectrum of β-lactamases (ESBLs), which inactivate new cephalosporins but which are still susceptible to β-lactamase inhibitors (eg. clavulanic acid)[5].

Currently, several methods such as the Bauer-Kirby test, minimal-inhibitory concentration (MIC), minimal bactericidal concentration (MBC), Serum-killing power, or PCR are being used for the detection of antibiotic-resistant bacteria[7]. However, these are somewhat tedious and require a long time span for detection. They also are limited in their assay volume and time. Therefore, it is necessary to develop a simple and rapid method that can accurately detect the antibiotic-resistant bacteria in many samples within a short time span to monitor and control therapeutic choice[8].

DNA chip is a very powerful technique used to detect DNA sequences or gene expression levels of thousands of genes by hybridization of thousands of DNA probes on a small material surface called a chip[9]. The applications can be used for the identification of genetic variations, genetic diseases, cancer diagnosis and more. This format permits the simultaneous monitoring and analysis of a large number of genetic features in one easy hybridization experiment[2].

The purpose of this study is to determine the availability of DNA chip system to detect the genes of *E coli* isolates from porcine fecal samples and to study the prevalence of β-lactam antibiotic-resistant genes.

Materials and Methods

The antibiotic-resistant genes on the DNA chip were *PSE, OXA, FOX, MEN, CMY, TEM, SHV, OXY* and *AmpC*, which were supplied by the Culture Collection of Antibiotics Resistant Microbes (Seoul, Korea). Four hundred *E coli* isolates from the diarrheic fecal samples of pigs during 2005-2006 were used in the study. The antibiotic susceptibilities of the *E coli* isolates were analyzed using agar dilution method proposed by the National Committee for Clinical Laboratory Standards (NCCLS). The following antibiotics were tested: ampicillin, cefazolin, cefoxitin, cefotaxime, and ceftriaxone (Sigma Chemical Co, St. Louis, Mo.); amoxicillin-

clavulanic acid (AMC) and ticarcillin (TIC; SmithKline Beecham, Madrid, Spain); ceftazidime (Glaxo, Madrid, Spain); imipenem (Merck Sharp & Dohme, Madrid, Spain); and aztreonam (Bristol-Myers Squibb, Madrid, Spain).

Table 1. The sequence of primers for each antibiotic resistant gene

	Primer sequence
*TEM	5'-ata aaa ttc ttg aag acg aa-3'
	5'-aca gtt acc aat gct taa tc-3'
SHV	5'-tgg tta tgc gtt ata ttc gc-3'
	5'-ggt tag cgt tcg cag tgc-3'
MEN	5'-tcc tct ctt cca ga-3'
	5'-cag cgc ttt tcg cgt cta a-3'
CMY	5'-atg caa caa cga caa tcc a-3'
	5'-gtt ggg tag ttg cga ttg g-3'
OXY	5'-cag atc tcg aga agc gtt c-3'
	5'-acc tct ttg cgg ttt ttc g-3'
FOX	5'-cac cac gcg aat aac cat-3'
	5'-atg tgg acg cct tga act-3'
PSE	5'-aat ggc att cag cgc ttc cc-3'
	5'-ggg gct tga tgc tca ctc c-3'
OXA	5'-tca act ttc aag atc gca-3'
	5'-gtg tgt tta gaa tgg tga-3'
AmpC	5'-cta cgg tct ggc tgc ta-3'
	5'-gtt ggg gta gtt gcg att g-3'

*Antibiotic resistant gene

The probe was prepared using a method described by Lee et al[2]. Each of the β-lactamase antibiotic-genes (*PSE*, *OXA*, *FOX*, *MEN*, *CMY*, *TEM*, *SHV*, *OXY*, and *AmpC*) were amplified using a primer set specific to each gene (Table 1). Each PCR constituted 30 cycles of denaturation, hybridization, and elongation. The DNA chip was fabricated by spotting the amplified probe DNAs in a spotting buffer (350 mM sodium bicarbonate, pH 9.0) at 1 μM concentration onto a glass slide that had been coated with the aldehyde group (CEL Associates, USA) by MicroGrid II (BioRobotics, UK). The amine group on the DNAs was coupled with the aldehyde group on a slide using the Schiff's base reaction. $NaBH_4$ was used to reduce the remaining aldehyde groups. This DNA chip also contained the yeast DNA fragment, S400, as a positive control, which was confirmed not to be cross-hybridized with the bacterial genes (Fig 1).

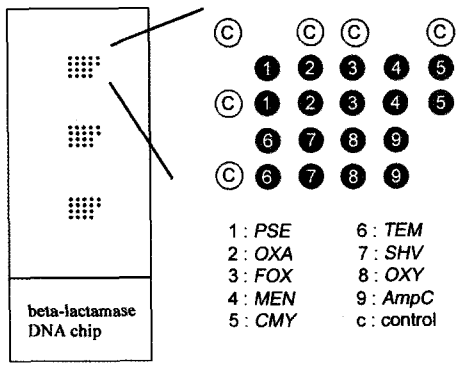

Fig 1. Design of the DNA chip for detecting beta-lactam antibiotics resistant genes

The positive control DNA was also labeled using the same procedure used for the target DNA, except that 200ng of the plasmid containing the yeast DNA fragment S400 was used. The 20μℓ target DNA and 1μℓ positive control were suspended in a 20μℓ hybridization solution. Amplification was carried out using the following program: an initial denaturing step of 95℃ for 10 minutes followed by 35 cycles of 95℃ for 30 seconds, 50℃ for 30 seconds, and 72℃ for 40 seconds, and a final extension at 72℃ for 10 minutes. The hybridization solution contained the target DNAs that had been denatured at 90℃ for 5 minutes, and then hybridized for

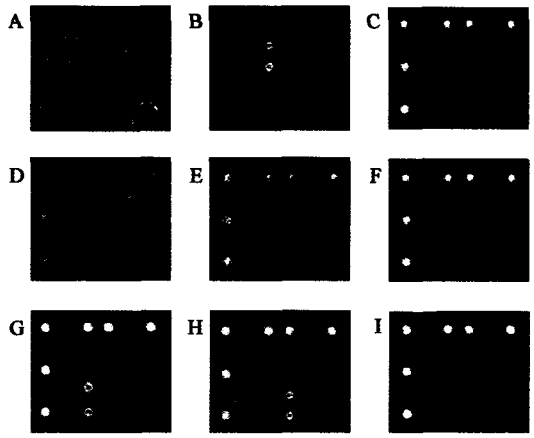

Fig 2. Laser scan images of DNA chips to which the cy5-labeled PCR amplified products of the reference β-lactam antibiotic-resistant genes from the *Escherichia coli* had been hybridized. A: *PSE*, B: *OXA*, C: *FOX*, D: *MEN*, E. *CMY*, F: *TEM*, G: *SHV*, H: *OXY*, and I: *AmpC*.

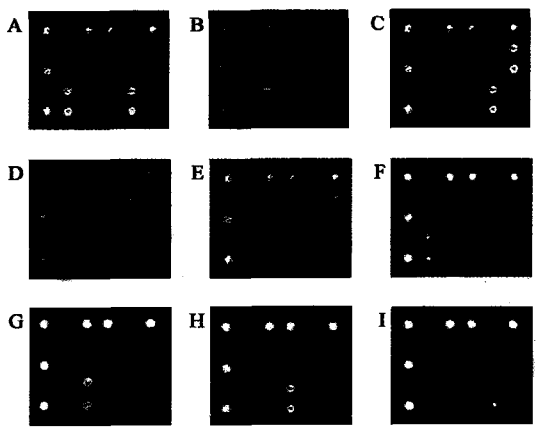

Fig 3. Laser scan images of DNA chips to which the cy5-labeled PCR amplified products of the β-lactam antibiotic-genes from the samples had been hybridized. A: *TEM* and *AmpC*, B: *SHV* and *AmpC*, C: *CMY* and *AmpC*, D: *MEN*, E. *CMY*, F: *TEM*, G: *SHV*, H: *OXY*, and I: *AmpC*.

for 1 hours at 70℃. The DNA chip was washed, and then scanned (ScanArray 5000, Packard, USA) and analyzed using the QuantArray software (Packard, USA).

Results and Discussion

Of the 400 *E coli* samples isolated from pig feces, 339 (85%) were resistant to the β-lactam antimicrobials used in this study, according to the Bauer-Kirby method for testing the antimicrobial sensitivity (data not shown). All of the isolated bacteria were resistant to penicillin, cephalonium, amoxicillin, amoxicillin/clauvanic acid, ampicillin, and cefotaxime. The bacteria were also found to be resistant to other types of antimicrobial agents such as erythromycin (100%), tetracycline (100%), rifampin (100%), colistin (100%), teicoplanin (100%), neomycin (97.5%), gentamycin (95%), trimethoprim/sulfamethoxazole (92.5 %), ciprofloxacin (60 %) and norfloxacin (57.5%).

It was assumed that resistance toward β-antibiotics in *E coli* isolated from pigs is on the increase in Korea. A study [10] conducted in 2,000 indicated that 78% of the *E coli* isolates were resistant to ampicillin and 44% were resistant to cephalothin, but resistant to ampicillin was 100% in this study.

Fig 2 and Fig 3 showed the results of the β-lactamase resistant genes by DNA chip. Three hundred and thirty-nine isolates had at least one β-lactamases gene. Resistance to β-lactamases was mediated mainly by *AmpC* (n=339, 100 %), and followed by *TEM* (n = 200, 59.0 %), *CMY* (n = 101, 29.8%), *PSE* (n=30, 8.9%) but no *FOX*, *MEN* and *OXY* genes were detected.

Among 339 isolates having β-gene, 70 (20.6%) were found to have three or more

resistant genes, while 190 (56.0%) had two resistant genes. Three were found to have four antibiotic resistant β-lactamase genes. They were detected with having *AmpC*, *TEM* and *CMY* genes; and *OXA* and two *PSE* genes, respectively (Fig 3). The other sixty-one did not have any β-genes even though they were found to be resistant to the antimicrobial drugs.

Beta-lactams are widely used in human and veterinary medicine to treat various infections[11]. In this study, the highest expression of the *AmpC* genes highlighted the significant role of these isolates in the resistance phenomena to β-lactams. Organisms overexpressing *AmpC* are a major clinical concern because they are usually resistant to many β-lactam drugs with the exception of cefepime, cefpirome, and the carbapenems[12]. The presence of more than one β-lactamase resistant gene in a single bacterium is also a great concern because most of the problems associated with resistance are usually related to the over use of broad-spectrum antibiotics in the livestock industry.

The increased use of third generation cephalosporins in humans was also followed by an increasing prevalence of extended-spectrum β-lactam-resistant Gram negative bacteria in the country[2]. The level of antimicrobial resistant bacteria in Korea is possibly among the highest in the world[13].

The possibility of new emerging β-lactamases genes in pigs in Korea should be taken seriously because the evolution and dissemination of antibiotic resistant genes is continuously occurring in animals, humans and agricultural hosts throughout the world. More work will be needed to identify the new emerging β-lactamases genes from animal clinical samples, which are important in veterinary public health. Gene exchange can occur in soil or more likely in gastrointestinal tract of animals or humans.

In humans, identifying organisms expressing the extended-spectrum β-lactamases (ESBLs) is essential for monitoring of hospital infections as well as for epidemiological studies in veterinary science. The DNA chip can be used to detect the expression of these resistance genes hence differentiate the different types of β-lactamases enzymes in pig fecal samples. The plasmid-mediated AmpC gene is commonly found in nosocomial human isolates of *E coli* and *Klebsiella pneumonia*, as well as in strains of other genera of the family *Enterobacteriaceae*[14].

In conclusion, the antibiotic-resistant detection system based on a DNA chip can be a rapid and reliable method for detecting of various resistant bacteria simultaneously in one reaction and it is possible to detect a single β-lactam antibiotic-resistant bacterium without culture. This system can also simultaneously detect various resistant genes in one reaction in a reproducible manner.

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