

# Molecular detection of *bla*<sub>VIM</sub>, *bla*<sub>BIC</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>SIM</sub> genes from isolated bacteria in retail meats

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## 육류용 고기로부터 분자진단을 이용한 항생제내성 유전자 양성

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**Abstract** The purpose of this study was to investigate the ability to treat and prevent infection by multiple Gram-negative bacterial pathogens as a last choice option in the treatment of serious infections in clinical settings. The global spread of extended-spectrum  $\beta$ -lactamases (ESBLs) and/or carbapenemases in microorganisms are of enormous concern to health services because they are often associated with multi-drug resistance which significantly restricts the antibiotic treatment options. In this study, the antimicrobial resistance profiles of bacteria isolated from South Korean market-derived meat samples were determined by the disc diffusion method. PCR was used to detect the presence of antibiotic resistance genes and ESBL producing genes. In total, we tested 181 isolated colonies from 36 market-derived meat samples. Single PCR and DNA sequencing results revealed that genes *bla*<sub>VIM</sub>, *bla*<sub>BIC</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>SIM</sub> were present in the bacteria isolated from retail meat. The bacteria in the meat were separately sequenced and based on alignment, four different bacteria were identified. These findings suggest that bacteria found in retail meats are a reservoir for the spreading of ESBL *bla*<sub>VIM</sub>, *bla*<sub>BIC</sub>, *bla*<sub>KPC</sub>, and *bla*<sub>SIM</sub> resistance genes and bacteria strains.

**요약** 본 연구의 목적은 확장 스펙트럼  $\beta$ -락타마제(ESBL) 항생제가 그람 음성 세균에 의한 감염을 치료하고 예방하는데 사용하고 있으며 임상에서 심각한 감염을 치료하는데 선택하는 마지막 옵션 역할을 한다. 미생물의 확장 스펙트럼  $\beta$ -락타마제(ESBL) 및/또는 카르바페네마제(Carbapenemase) 내성에 대한 보고는 전 세계적으로 확산되는 것으로 보고되며 항생제 치료에 많은 제한을 주는 요인으로 다약제 내성과 관련이 있기 때문에 보건 서비스에 큰 관심을 가지고 있다. 본 연구는 국내 시장에서 구매하여 분리한 육류로부터 세균을 분리 동정하여 항생제 저항성 테스트인 디스크 확산법을 사용하여 내성균을 분리 실험하였고, PCR과 DNA 시퀀싱방법을 수행하였다. 결과는 PCR을 수행하여 항생제 내성 유전자와 유전자를 생산하는 ESBL의 존재를 검출하고 결과를 얻었다. 총 36개의 샘플 육류로부터 181개의 각각 분리된 세균을 추출하여 실험결과를 얻었다. 결과는 PCR과 DNA 염기서열을 분석하여 항생제내성 유전자로 *bla*<sub>VIM</sub>, *bla*<sub>BIC</sub>, *bla*<sub>KPC</sub>, *bla*<sub>SIM</sub>으로 나타났다. 분리한 육류 속의 박테리아는 별도 유전자 서열분석으로 4개의 다른 박테리아가 확인되었다. 이러한 결과는 소매되는 육류에서 발견되는 박테리아에 ESBL 내성유전자인 *bla*<sub>VIM</sub>, *bla*<sub>BIC</sub>, *bla*<sub>KPC</sub>, *bla*<sub>SIM</sub>를 가진 박테리아 균주가 있을 수 있으며 이는 특수 확장 스펙트럼  $\beta$ -락타마제(ESBL) 및/또는 카르바페네마제(Carbapenemase) 내성유전자가 확산될 수 있다는 것을 시사한다.

**Keywords** : Carbapenemase, Retail Meats, Extended-Spectrum  $\beta$ -lactamases (ESBLs), Multi-Drug Resistance (MDR), Antimicrobial Susceptibility

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## 1. Introduction

Currently, more than 1,300 distinct  $\beta$ -lactamases have been identified in clinical isolates, and among these, extended-spectrum  $\beta$ -lactamases (ESBLs) are considered the most deleterious due to their ability to hydrolyze most penicilins and cephalosporins [1]. ESBLs antibiotics have the ability to prevent or treat multiple Gram-negative bacterial infections, and are used as a first-line of therapy against ESBL-producing *Enterobacteriaceae* [2]. Unfortunately, the incidence of ESBL resistance has increased worldwide during the last few years, especially among Gram-negative bacterium such as *Pseudomonas spp.*, and *Acinetobacter spp.*, as well as *Enterobacteriaceae* [3]. There are two main mechanisms of resistance, including the acquisition of ESBL genes encoding for enzymes which can degrade ESBLs, or reducing the uptake of antibiotics by changing the permeability of the membrane [4]. The most important ESBLs fall into three classes of enzymes: class A ESBLs (KPC and BIC types), class B metallo-enzymes (VIM, IMP, SPM, NDM, AIM, DIM, GIM and SIM types), and class D enzymes (OXA-48 type) [5, 6]. Recent studies have revealed that KPC, NDM and OXA-48 are currently the most clinically significant carbapenemases, and the majority of corresponding genes are carried by plasmid and associated with various mobile genetic elements, which play an important role in spread of ESBL resistance genes [4, 7].

Some of these bacteria can spread rapidly between humans via different pathways, such as the hands, contaminated food or water, and materials used in hospital settings [8]. In addition, there are an increasing number of reports indicating the presence of carbapenemase-producing microorganisms among non-human sources, including food-producing animals and their environment, as well as in companion animals [3, 9, 10]. To the best of our knowledge,

there is as yet, no study demonstrating carbapenemase-producing microorganisms on retail meat or other foodstuffs, but the potential for such reservoirs should be investigated.

In our study were determined antimicrobial susceptibility and existence of ESBL resistance genes screening among bacteria isolated from retail meats. I have investigated the role that animal origin food products play in delivering ESBL resistance genes and bacteria strains.

## 2. Materials and methods

### 2.1 Sample collection and bacterial isolation

A total of 31 bacteria strains were collected from 38 poultry and livestock meat samples, including pork (n=18), beef (n=12), chicken (n=6) and duck (n=2), taken from 17 different markets (Table 1). These samples were purchased from production regions and obtained without the possibility of infection in the home. Sample strains were streaked onto Luria-Bertani(LB) agar culture (Sinyang Diagnostics, Seoul, Korea). Single colony was picked from each LB agar plate and incubated in LB broth with shaking (80 rpm) at 37 °C overnight. Bacterial identification was based on 27F and 1492R primer sets for 16s rRNA gene.

### 2.2 Antimicrobial Susceptibility Testing

We tested for antimicrobial susceptibility using the Kirby-Bauer disc diffusion method described by Clinical and Laboratory Standard Institute (CLSI) guidelines, 2013 [11]. Each 31 bacterial suspension was adjusted to McFarland 0.5 turbidity, swabbed onto Mueller-Hinton agar, and incubated in the presence of antibiotic discs at 37 °C for 18 hr. Antimicrobial susceptibility test was performed according to an existing Mun's procedure [Mun & Hwang, 2019, 12].

### 2.3 Multiplex PCR for detection of ESBL resistance genes

Genes *bla*<sub>AMP</sub>, *bla*<sub>SPM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub>, *bla*<sub>BIC</sub>, *bla*<sub>NDM</sub>, *bla*<sub>AIM</sub>, *bla*<sub>GIM</sub>, *bla*<sub>SIM</sub> and *bla*<sub>DIM</sub> were detected by multiplex PCR using specific primers listed in reference [13, 14]. Multiplex PCR improves screening efficiency for antibiotic resistance gene detection. Three multiplex PCR reactions were designed, with group I including primers for detection of *bla*<sub>AMP</sub>, *bla*<sub>SPM</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>OXA-48</sub> genes; group II including detection for *bla*<sub>KPC</sub>, *bla*<sub>BIC</sub> and *bla*<sub>NDM</sub> genes; and group III including detection for *bla*<sub>AIM</sub>, *bla*<sub>GIM</sub>, *bla*<sub>SIM</sub> and *bla*<sub>DIM</sub> genes. The multiplex PCR mixtures for the detection of each gene group contained 10 pmol of each primer, 10  $\mu$ l iQ<sup>TM</sup> SYBR<sup>®</sup> Green supermix (2 $\times$  reaction buffer with dNTPs, iTaq DNA polymerase, MgCl<sub>2</sub>, SYBR<sup>®</sup> Green I, fluorescein, and stabilizers). The volume was made to 20  $\mu$ l by addition of autoclaved triple-distilled water and then a small amount of colony was added to each PCR reaction. DNA amplification was carried out using the thermal cycler, with cycling conditions: 94  $^{\circ}$ C for 10 min and 40 cycles at 94  $^{\circ}$ C for 45 s, 56  $^{\circ}$ C for 40 s and 72  $^{\circ}$ C for 50 s, with a final extension step at 72  $^{\circ}$ C for 5 min. A 100 bp DNA ladder was used as molecular size marker on the gels. PCR products were subjected to electrophoresis in 2 % agarose gel in 1 $\times$  TBE buffer at 100 V for 20 min and visualized with Safe Green loading dye.

### 2.4 Single PCR for detection of ESBL genes

Based on the results of multiplex PCR, single PCR was carried out to further confirm the existence of extended-spectrum  $\beta$ -lactamase resistance genes in bacterial strains. The multiplex PCR detected strains were used as template DNA and single PCR mixtures contained 10 pmol of each primer, 2  $\mu$ l DNA (100 ng), 10  $\mu$ l iQ<sup>TM</sup> SYBR<sup>®</sup> Green supermix (2 $\times$  reaction buffer with dNTPs, iTaq DNA polymerase, MgCl<sub>2</sub>,

SYBR<sup>®</sup> Green I, fluorescein, and stabilizers). The volume was made to 20  $\mu$ l by addition of autoclaved triple-distilled water. DNA amplification was carried out using thermal cycler, with cycling conditions: 94  $^{\circ}$ C for 3 min and 36 cycles at 94  $^{\circ}$ C for 30 s, 62  $^{\circ}$ C for 30 s and 72  $^{\circ}$ C for 45 s, with a final extension step at 72  $^{\circ}$ C for 5 min. The purpose of using 62 $^{\circ}$ C for annealing temperature was to selectively amplify certain detection sites. A 100 bp DNA ladder was used as molecular size marker on the gels. PCR products were subjected to electrophoresis in 2 % agarose gel in 1 $\times$  TBE buffer at 100 V for 20 min and visualized with Safe Green loading dye.

### 2.5 DNA sequencing and alignments

Bacteria were classified by 16s rRNA criteria for 25 isolates. Samples No's 4, 6, 8, 10, 11, and 13 bacteria were used for identify by alignment. Single PCR products of *bla*<sub>SPM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>BIC</sub>, *bla*<sub>KPC</sub>, *bla*<sub>AIM</sub>, *bla*<sub>SIM</sub> and 16s rRNA were sequenced on an A3730 sequencer (ABI, Foster city, CA, USA). Sequence alignments were performed using BLASTN, Pubmed (<https://blast.ncbi.nlm.nih.gov/Blast>).

## 3. Results

### 3.1 Bacterial identification and antimicrobial resistances

Bacteria were classified by 16s rRNA criteria for 25 isolates. The bacteria in the meat were separated and four different bacteria were identified. I have measured the diameters of inhibition zones  $\leq$ 10-13 mm and determined each isolate as resistant or susceptible to antimicrobial agents based on CLSI 2015 and Liofilchem (Liofilchem, Roseto degli Abuzzi, Italy). The susceptibility test showed that 42.1% (16/38) of poultry and livestock meat samples were resistant to ampicillin. The results also showed high rates of resistance to tetracycline

Table 1. Bacterial colony detection from purchased Domestic and Imported meats

Sample No.	Types	Sub-colony selection No.	Region	Purchase date	ETC
1	Beef	6	Australia(IM)	2014.12.22	
2	Pork	6	Gyenggido	2015.01.03	
3	Duck	6	Jeollanamdo	2015.01.09	
4	Pork	8	Jeju	2015.01.08	
5	Beef	8	Chungcheongbuk-do Eumseong Gun	2015.01.10	
6	Chicken	6	Jeollabuk-do Jeongeub	2015.01.11	
7	Beef	10	Chungcheongbuk-do Eumseong Gun	2015.01.07	
8	Pork	4	Jeju	2015.01.11	
9	Chicken	6	Jeollabuk-do Iksan	2015.01.07	
10	Beef	6	Gyeongsangbuk-do Mungyung	2014.11.08	
11	Pork	2	DM ChungcheonNamdo	2015.01.17	
12	Pork	6	ChungcheonNamdo Hongseong	2015.01.14	
13	Pork	6	DM ChungcheonNamdo	2015.01.17	
14	Pork	8	Gyeonggi-do Kimpo	2015.01.17	
15	Pork	4	Jeollabuk-do Kimje	2015.01.18	
16	Chicken	8	Jeollabuk-do Iksan	2015.01.21	
17	Pork	10	Chungcheongbuk-do Eumseong Gun	2015.01.23	
18	Beef	3	Gyeongsangbuk-do Goyeong	2015.01.22	
19	Pork	4	Chungcheongbuk-do Eumseong Gun	2015.01.25	
20	Beef	6	Jeollanamdo Damyoang	2015.01.20	
21	Beef	4	ChungcheonNamdo Hongseong	2015.01.25	
22	Beef	-	IM	2015.01.08	PD
23	Beef	-	IM	2015.01.08	PD
24	Pork	-	IM	2015.01.08	PD
25	Pork	-	IM	2015.01.08	PD
26	Duck	6	Jeollanamdo	2015.02.07	
27	Beef	6	DM	2015.02.14	
28	Beef	8	Jeju	2015.02.11	
29	Pork	4	DM Costoco	2015.02.28	
30	Pork	2	Chungcheongbuk-do Chungju	2015.03.07	
31	Beef	4	Chungcheongbuk-do Jecheon	2015.03.07	
32	Pork	6	ChungcheonNamdo Dangjin	2015.03.07	
33	Pork	6	DM	2015.03.21	
34	Chicken	4	DM	2015.03.21	
35	Beef	6	Chungcheongbuk-do Eumseong Gun	2015.03.20	
36	Chicken	4	Jeollabuk-do Jeongeub	2015.03.20	
37	Beef	6	Jeollanamdo Suncheon	2015.03.12	
38	Pork	2	Jeollanamdo Suncheon	2015.02.14	

\* IM: imported manufactured meat product. Others: local farm

\* DM: domestic manufactured meat

55.3% (21/38) and kanamycin 36.8% (14/38), however, most of the samples were susceptible to gentamycin with only 5.3% (2/38) resistant.

### 3.2 Detection of Multiplex PCR and single PCR products for ESBL resistance genes

Amplification products of group I, group II and group III multiplex PCR were obtained and all of the products were separated by agarose gel electrophoresis (Fig. 1). To increase specific amplification, single PCRs were also performed based on the multiplex PCR results. The results in group I multiplex-positive PCRs showed that high-yield 390 bp *bla*<sub>VIM</sub> specific amplification products were detected in strains NO.8 and NO.13-3. In addition, *bla*<sub>VIM</sub> specific amplification products were also found in strains NO.6 and NO.6-2. The results in group II multiplex-positive PCRs confirmed that 232 bp *bla*<sub>KPC</sub> specific amplification products were detected in strain NO.6. In addition, 537 bp *bla*<sub>BIC</sub> specific amplification products were also found in strains NO.13-1 and NO.13-2. Results from Group III multiplex-positive PCRs demonstrated that 570 bp *bla*<sub>SIM</sub> specific amplification products were detected in strain NO.11-3 (Fig. 1, Table 2). Other products seen in the multiplex data, did not however, show specific amplification. This demonstrated that non-specific amplification products were seen not only in the group I multiplex PCR, but also in groups II and III as well.

### 3.3 Comparison of DNA sequence and alignments

Single gene PCR showed that genes *bla*<sub>VIM</sub>, *bla*<sub>BIC</sub>, *bla*<sub>KPC</sub> and *bla*<sub>SIM</sub> were present in bacteria isolated from retail meat (Fig. 1). Although we obtained single PCR products of the expected size, sequence alignments revealed that four samples of them are target genes. extended-spectrum  $\beta$ -lactamases genes were detected 4 samples in our strains.

Bacteria were classified by 16s rRNA criteria

for 25 isolates. Four different bacteria were identified by alignment. No's 4, 6, 8, 10, 11, and 13 were identified as belonging to one of the four species. No. 4. #K-4-2-4-1: *Kocuria rhizophila* (Kovács et al. 1999) 99.29%. No. 6. #T-6-0-3-4: *Escherichia coli* (Migula 1895; Castellani and Chalmers 1919) 100%. No. 8. #T-8-0-5-3: *Macrococcus caseolyticus* (Schleifer et al. 1982; Kloos et al. 1998) 99.76%. No. 10, 11, and 13. #K-10-0-1-5: *Staphylococcus warneri* (Kloos and Schleifer 1975) 100% (Table 2).

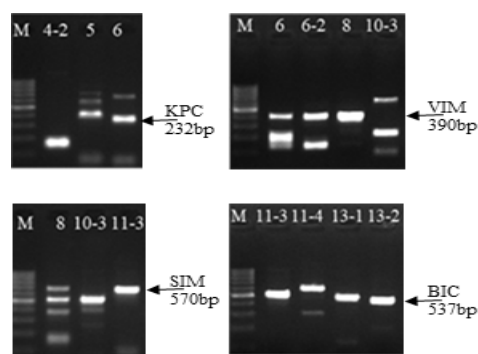


Fig. 1. Results of single PCR for amplification of extended-spectrum  $\beta$ -lactamases genes. The PCR products are separated on a 2% agarose gel. Lane M, molecular size marker. Sample names are indicated on the top of figures. No 6 detected Single PCR for *bla*<sub>KPC</sub> (232/798 bp), No 8 detected Single PCR for *bla*<sub>VIM</sub> (390 bp), No 11-3 detected Single PCR for *bla*<sub>SIM</sub> (570 bp). No 13-1 & 13-2 detected Single PCR for *bla*<sub>BIC</sub> (537 bp).

Table 2. Detection results of single PCR for amplification of extended-spectrum  $\beta$ -lactamases genes.

Bacteria Name	Sample No.	Sub-cloning No.	Gene
<i>Kocuria rhizophila</i>	4	#K-4-2-4-1	Not Detect
<i>Escherichia coli</i>	6	#T-6-0-3-4	KPC(6) VIM(6-2)
<i>Macrococcus caseolyticus</i>	8	#T-8-0-5-3	VIM(8)
<i>Staphylococcus warneri</i>	10 11 13	#K-10-0-1-5	SIM(11-3) BIC(13-1,13-2) VIM(13-3)

\* Detection results of single PCR for amplification of extended-spectrum  $\beta$ -lactamases genes. Sample names are indicated on the Bacteria name. No 6 detected for *bla*<sub>KPC</sub>. No 8 detected for *bla*<sub>VIM</sub>. No 11-3 detected for *bla*<sub>SIM</sub>. No 13-1 & 13-2 detected for *bla*<sub>BIC</sub>.

## 4. Discussion

There are an increasing number of reports which indicate the presence of ESBL genes present in bacteria isolated from non-human sources. However, at present there is no research on ESBL-acquiring microorganisms on retail meat or other foodstuffs in South Korea.

Although ESBL genes were small quantities detected in bacteria isolated from retail meat in our study, a lot of quantities detectable in the possibility needs to be considered. There are an increasing number of reports which indicate the presence of ESBLs among bacteria isolated from non-human sources. For example, it has been reported that VIM-1 ESBLs was present in *Salmonella* and *E. coli* strains isolated from food producing animals in Germany. Clearly, once these bacteria are maintained in a farming environment, they may become widely dispersed among animals [15, 16]. The dissemination of ESBL producing organisms represents a serious public health threat because of the associated multi-drug resistance which significantly narrows the antibiotic treatment options. At present there are limited reports of ESBL genes in bacteria from retail meat, so our findings raise the alarming prospect that animal origin food products may represent a potential source of antibiotic resistant organisms.

Gram-negative antibiotic resistant genes can be transferred between different strains, species and genera by mobile genetic elements. As an example which appeared in this study, the gene *bla<sub>KPC</sub>* in *K. pneumoniae* has been reported on many plasmids, and the worldwide spread of *bla<sub>KPC</sub>* genes are associated with a mobile genetic element (transposon *TnA401*), which can jump to numerous conjugative plasmids. One of the common genes harbored by these plasmids is the *tra* operon, which encodes plasmid conjugation machinery proteins and may play an important role in the successful dissemination of *bla<sub>KPC</sub>*-harboring plasmids [17]. The horizontal transfer of *bla<sub>KPC</sub>*

among *Enterobacteriaceae* which colonise the human intestine is frequent, and plasmid encoded *bla<sub>KPC</sub>* gene can be transferred from *K. pneumoniae* to *E. coli* [18, 19].

The ESBL-producing microorganisms identified here, are not common food-borne or animal pathogens, therefore it is likely they would elude common resistance surveillance programs [20]. A major question to consider therefore, is would retail meats that tested positive for ESBL bacteria be considered fit for human consumption? Ingesting improperly cooked retail meat can present significant health risks because it provides an opportunity for the transfer of ESBL genes to resident bacteria during handling. Therefore effective surveillance for ESBL producing bacteria in the food chain is urgently required, and the prevalence of ESBL gene-containing bacteria should be further investigated.

## 5. Conclusion

ESBL genes were detected in four bacterial species isolated from retail meat. Single PCR and DNA sequencing results revealed that genes *bla<sub>VIM</sub>*, *bla<sub>BIC</sub>*, *bla<sub>KPC</sub>* and *bla<sub>SIM</sub>* were present.

The emergence of ESBL genes in retail meat presents a significant potential public health threat. Ingestion of improperly cooked meat permits the transfer of ESBL genes to resident bacteria and speeds the transmission of ESBL genes for which few therapeutic options exist.

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(Research Interests)

Cancer & Micro environment Molecular Imaging, Functional natural substances.