Molecular detection of $\text{bla}_{\text{VIM}}$, $\text{bla}_{\text{BIC}}$, $\text{bla}_{\text{KPC}}$, and $\text{bla}_{\text{SIM}}$ genes from isolated bacteria in retail meats

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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 $\text{bla}_{\text{VIM}}$, $\text{bla}_{\text{BIC}}$, $\text{bla}_{\text{KPC}}$, and $\text{bla}_{\text{SIM}}$ 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 $\text{bla}_{\text{VIM}}$, $\text{bla}_{\text{BIC}}$, $\text{bla}_{\text{KPC}}$, and $\text{bla}_{\text{SIM}}$ resistance genes and bacteria strains.

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

Received April 13, 2021 Revised May 20, 2021 Accepted June 4, 2021 Published June 30, 2021
1. Introduction

Currently, more than 1,300 distinct β-lactamases have been identified in clinical isolates, and among these, extended-spectrum β-lactamases (ESBLs) are considered the most deleterious due to their ability to hydrolyze most penicillins 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, 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 we 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). Theses 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 blaIMP, blaSPM, blaVIM, blaOXA-48, blaKPC, blaBIC, blaNDM, blaAIM, blaGIM, blaSIM and blaDIM 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 blaIMP, blaSPM, blaVIM, and blaOXA-48 genes; group II including detection for blaKPC, blaBIC and blaNDM genes; and group III including detection for blaAIM, blaGIM, blaSIM and blaDIM genes. The multiplex PCR mixtures for the detection of each gene group contained 10 pmol of each primer, 10 μl iQ™ SYBR® Green supermix (2× reaction buffer with dNTPs, iTaq DNA polymerase, MgCl₂, SYBR® Green I, fluorescein, and stabilizers). The volume was made to 20 μl by addition of autoclaved triple-distilled water. DNA amplification was carried out using thermal cycler, with cycling conditions: 94 ℃ for 10 min and 40 cycles at 94 ℃ for 30 s, 62 ℃ for 30 s and 72 ℃ for 45 s, with a final extension step at 72 ℃ for 5 min. The purpose of using 62℃ 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× 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 β-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 μl DNA (100 ng), 10 μl iQ™ SYBR® Green supermix (2× reaction buffer with dNTPs, iTaq DNA polymerase, MgCl₂, SYBR® Green I, fluorescein, and stabilizers). The volume was made to 20 μl by addition of autoclaved triple-distilled water. DNA amplification was carried out using thermal cycler, with cycling conditions: 94 ℃ for 3 min and 36 cycles at 94 ℃ for 30 s, 62 ℃ for 30 s and 72 ℃ for 45 s, with a final extension step at 72 ℃ for 5 min. The purpose of using 62℃ 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× 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 blaSPM, blaNDM, blaBIC, blaKPC, blaAIM, blaSIM 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 ≤10-13 mm and determined each isolate as resistant or susceptible to antimicrobial agents based on CLSI 2015 and Liofilchem (Liofilchem, Roseto degli Abruzzi, 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

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Types</th>
<th>Sub-colony selection No.</th>
<th>Region</th>
<th>Purchase date</th>
<th>ETC</th>
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<tbody>
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<td>6</td>
<td>Australia(IM)</td>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
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</tr>
<tr>
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<tr>
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<td>6</td>
<td>Jeollalbuk-do Jeongeub</td>
<td>2015.01.11</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>8</td>
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<td>4</td>
<td>Jeju</td>
<td>2015.01.11</td>
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</tr>
<tr>
<td>9</td>
<td>Chicken</td>
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<td>Jeollalbuk-do Iksan</td>
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<td>38</td>
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<td>2</td>
<td>JeollaNamdo Suncheon</td>
<td>2015.02.14</td>
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</tbody>
</table>

* 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 blavim-specific amplification products were detected in strains NO.8 and NO.13-3. In addition, blavim 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 blakpc specific amplification products were detected in strain NO.6. In addition, 537 bp blabic 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 blaSIM 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 blavim, blabic, blakpc and blaSIM 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 β-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: Macrooccus 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).

Table 2. Detection results of single PCR for amplification of extended-spectrum β-lactamases genes.

<table>
<thead>
<tr>
<th>Bacteria Name</th>
<th>Sample No.</th>
<th>Sub-cloning No.</th>
<th>Gene</th>
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</thead>
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<td>Kocuria rhizophila</td>
<td>4</td>
<td>#K-4-2-4-1</td>
<td>Not Detect</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>6</td>
<td>#T-6-0-3-4</td>
<td>KPC(60)  VIM(6-2)</td>
</tr>
<tr>
<td>Macrooccus caseolyticus</td>
<td>8</td>
<td>#T-8-0-5-3</td>
<td>VIM(8)</td>
</tr>
<tr>
<td>Staphylococcus warneri</td>
<td>10</td>
<td>#K-10-0-1-5</td>
<td>SIM(11-3) BIC(13-1,13-2) VIM(13-3)</td>
</tr>
</tbody>
</table>

* Detection results of single PCR for amplification of extended-spectrum β-lactamases genes. Sample names are indicated on the Bacteria name. No 6 detected for blavim. No 8 detected for blakpc. No 11-3 detected for blaSIM. No 13-1 & 13-2 detected for blabic.
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 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 blaKPC in K. pneumoniae has been reported on many plasmids, and the worldwide spread of blaKPC genes are associated with a mobile genetic element (transposon Tn4401), 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 blaKPC-harboring plasmids [17]. The horizontal transfer of blaKPC among Enterobacteriaceae which colonise the human intestine is frequent, and plasmid encoded blaKPC 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 blaVIM, blaBIC, blaKPC and blaSIM 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.

References


Molecular detection of blaVIM, blaBIC, blaKPC, and blaSIM genes from isolated bacteria in retail meats


<Research Interests>
Cancer & Micro environment Molecular Imaging, Functional natural substances.