Chromosomal cephalosporinase in Enterobacter hormaechei as an ancestor of ACT-1 plasmid-mediated AmpC β-lactamase

Kyoung Ho Roh, Wonkeun Song, Hae-Sun Chung, Yang Soon Lee, Jong Hwa Yum, Ha Na Yi, Jong Sik Chun, Dongeun Yong, Kyungwon Lee and Yunsop Chong

1Department of Laboratory Medicine, Korea University College of Medicine, Seoul, Republic of Korea
2Department of Laboratory Medicine, Hallym University College of Medicine, Seoul, Republic of Korea
3Department of Laboratory Medicine, Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul, Republic of Korea
4Department of Clinical Laboratory Science, Dong-eui University, Busan, Republic of Korea
5School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul, Republic of Korea

In this study of the diversity of AmpC β-lactamase in clinical isolates of Enterobacter spp., a strain was found carrying the plasmid-mediated AmpC β-lactamase ACT-1 gene on its chromosome. The strain was identified as Enterobacter hormaechei using phylogenetic analysis of 16S rRNA and hsp60 genes. In addition, the species was confirmed by DNA–DNA hybridization. The genetic environment of the blaACT-1 gene was characterized, including the ampR and ampG genes, using a two-step PCR. The amino acid sequences of AmpR at serine 35, arginine 86, glycine 102, aspartic acid 135 and tyrosine 264 were conserved. Measurement of the transcription level of the blaACT-1 gene using real-time quantitative PCR showed that it increased 1.98-fold following cefotaxin induction. These results suggest that the plasmid-mediated blaACT-1 gene originated from the chromosome of E. hormaechei.

INTRODUCTION

Cephamycin resistance in organisms such as Enterobacter cloacae, Citrobacter freundii and Serratia marcescens can be caused by the induction or hyperproduction of AmpC β-lactamase (Livermore et al., 2001). Induction of this enzyme requires three additional gene products, AmpG, AmpR and AmpD (Reisbig et al., 2003). AmpG is a permease that transports muropeptides into the cytoplasm during cell-wall recycling. These peptides cause a conformational change in AmpR, resulting in induction of ampC transcription. Mutations in AmpD lead to derepression of the chromosomal ampC gene (Reisbig et al., 2003).

Initially, AmpC β-lactamase was chromosomally encoded; it expanded its host range by incorporating the genes into plasmids. Whilst most plasmid-mediated AmpC β-lactamases (PABLs) are expressed constitutively, the blaACT-1, blaDHA-1, blaDHA-2 and blaCMY-13 genes are reported to be inducible by the ampR genes upstream of ampC (Jacoby, 2009). Among these PABLs, ACT-1 was found initially in imipenem-resistant Klebsiella pneumoniae with the loss of an outer-membrane protein (Bradford et al., 1997). Although an ancestral association of ACT-1 with Enterobacter asburiae was proposed, the nucleotide and amino acid sequence identities were only 96.5 and 98 %, respectively (Rottman et al., 2002).

Currently, the taxonomy of the E. cloacae complex is based on whole-genome DNA–DNA hybridization and phenotypic characteristics (Brenner et al., 1986). E. asburiae, E. cloacae, Enterobacter hormaechei, Enterobacter kobei, Enterobacter ludwigii and Enterobacter nimipressuralis have been assigned to this complex. Accurate species identification is desirable because these organisms are increasingly isolated as nosocomial pathogens and are often not identified further to species level, perhaps because of the difficulty of achieving this (Paauw et al., 2008). The heat-shock protein 60 (hsp60) gene has been proposed as a promising marker for identification of Enterobacter spp. (Paauw et al., 2008).

Abbreviations: RT-qPCR, real-time quantitative PCR; PABL, plasmid-mediated AmpC β-lactamase.

The GenBank/EMBL/DDBJ accession numbers for the sequences of 16S rRNA, ampG, ampR, ampC, blc and sugE genes reported in this study are HQ215203, HQ215206 and HQ235645.
While determining the diversity of AmpC β-lactamases in *Enterobacter* strains isolated from clinical specimens, we found a strain carrying the bla<sub>ACT-1</sub> gene on its chromosome. The strain was identified as *E. hormaechei* using nucleotide sequencing of the 16S rRNA and hsp60 genes. In addition, DNA–DNA hybridization was performed to confirm the species. We characterized the genetic environment of the bla<sub>ACT-1</sub> gene because its induction was not observed in disk approximation tests. We also measured the transcription level of the bla<sub>ACT-1</sub> gene following cefoxitin induction using a cephalothin hydrolysis assay and real-time quantitative PCR (RT-qPCR). We propose that the plasmid-mediated bla<sub>ACT-1</sub> gene originated from the chromosome of *E. hormaechei*.

**METHODS**

**Bacterial strain and antimicrobial susceptibility testing.** An *Enterobacter* strain (YMC/KN/03/21) was isolated from urine collected from an intensive care unit patient in a Korean teaching hospital. Species identification and antimicrobial susceptibility tests were performed using the VITEK2 GN system (bioMérieux). Clinical and Laboratory Standards Institute guidelines (CLSI, 2010) were used to interpret the results.

**Phenotypic detection and spectrophotometric assay for ACT-1 induction.** Inducibility was screened using cefoxitin disk approximation tests with ceftazime, ceftazidime and aztreonam disks (Song et al., 2006) because cefoxitin is a strong inducer and is stable against hydrolysis. Cephalothin hydrolysis was assayed using crude enzyme extract obtained from a sonicated suspension following a 2 h induction assay with 10 μg cefoxitin ml<sup>-1</sup> (Sanders et al., 1986). The change in absorbance resulting from the opening of the β-lactam ring was measured using a UV-1601PC spectrophotometer (Shimadzu).

**16S rRNA and hsp60 gene sequencing and DNA–DNA relatedness.** 16S rRNA gene sequencing was performed using primers 8F and 1541R (Table 1) (Zhou et al., 1995) and the results were analysed using the EzTaxon server (Chun et al., 2007) and MEGA software (http://www.megasoftware.net). The primers for PCR and sequencing of the hsp60 gene were Hsp60 For and Hsp60 Rev (Table 1; Hoffmann & Roggenkamp, 2003). The DNA–DNA relatedness between strain YMC/KN/03/21 and the type strains of *E. hormaechei* CCUG 27126<sup>T</sup> and *E. asburiae* CCUG 25714<sup>T</sup> were evaluated using DNA–DNA hybridization. The DNA–DNA similarity value was calculated using the equation given by De Ley et al. (1970). A threshold value of 70 % DNA–DNA similarity was used to define bacterial species.

**Measurement of mRNA levels of the bla<sub>ACT-1</sub> gene by RT-qPCR.** Five colonies of clinical isolates of YMC/KN/03/21 were cultured in 10 ml Luria–Bertani broth overnight at 35 °C. A 2 h induction assay with cefoxitin (Sanders et al., 1986) was performed with 5 ml Luria–Bertani culture. Total RNA was extracted using an RNeasy Protect Bacteria Mini kit and an RNase-free DNAse Set (Qiagen) according to the manufacturer’s instructions. An Omniscript RT kit (Qiagen) with random primers (Promega) and RNase inhibitor (GenDEPOT) were used to produce cDNA. Primers Act1 F and Act1 R were then used for amplification, together with the Act1 probe (Table 1). The 5’ and 3’ ends of the probe were labelled with 6-carboxyfluorescein (FAM) and a fluorescence quencher dye (BHQ1), respectively. A capillary real-time thermal cycler (LightCycler; Roche Diagnostics) was used. The reaction conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 50 °C for 30 s and 72 °C for 1 min, with cooling for 30 min at 40 °C. Expression levels of the bla<sub>ACT-1</sub> gene with or without cefoxitin induction were normalized against expression of the 16S rRNA gene (using primers 16S rRNA F and 16S rRNA R and the 16S rRNA probe; Table 1) and compared.

**Sequencing of the AmpC β-lactamase, ampR and ampG genes.** AmpC β-lactamase genes were investigated using multiplex PCR (Pérez-Pérez & Hanson, 2002). The nucleotide sequences and deduced amino acid sequences were compared with sequences in the EMBL-EBI database (http://www.ebi.ac.uk/Tools/ss/fasta/). Two-step PCR was performed to determine the flanking nucleotide sequences in the upstream and downstream regions of the ampC gene (Sørensen et al., 1993), because induction of the ACT-1 enzyme was not observed in

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Purpose</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>8F</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>Species identification</td>
<td>Zhou et al. (1995)</td>
</tr>
<tr>
<td>1541R</td>
<td>AAGGAGGTGTCTACCACCC</td>
<td>Species identification</td>
<td>Zhou et al. (1995)</td>
</tr>
<tr>
<td>Hsp60 For</td>
<td>GTTAGAAGAGCCCCTTCGGTGTCGC</td>
<td>Species identification</td>
<td>Hoffmann &amp; Roggenkamp (2003)</td>
</tr>
<tr>
<td>Hsp60 Rev</td>
<td>ATGCAATTCTCGGTGATCATCATCAG</td>
<td>Species identification</td>
<td>Hoffmann &amp; Roggenkamp (2003)</td>
</tr>
<tr>
<td>Act1 F</td>
<td>CTGGGAATGTCTAAC</td>
<td>RT-qPCR sense</td>
<td>This study</td>
</tr>
<tr>
<td>Act1 R</td>
<td>GTTGAGATTCTACTTCTC</td>
<td>RT-qPCR antisense</td>
<td>This study</td>
</tr>
<tr>
<td>Act1 probe</td>
<td>AACCTATTGCTGGACTTCAACC</td>
<td>RT-qPCR probe</td>
<td>This study</td>
</tr>
<tr>
<td>16S RNA F</td>
<td>AGCTAGTAGTATGGAGAGGAGGGG</td>
<td>RT-qPCR sense</td>
<td>This study</td>
</tr>
<tr>
<td>16S RNA R</td>
<td>TTGGATTCCAGGTGATCAT</td>
<td>RT-qPCR antisense</td>
<td>This study</td>
</tr>
<tr>
<td>16S RNA</td>
<td>TCACCTTCCGTTGCTTCTCCAGA</td>
<td>RT-qPCR probe</td>
<td>This study</td>
</tr>
<tr>
<td>R0</td>
<td>TTGAAGAGCTTGTACCAAG</td>
<td>Two-step PCR</td>
<td>Sørensen et al. (1993)</td>
</tr>
<tr>
<td>R1</td>
<td>CAGTTCAACGTGTGTCAG flanking ampR ampG</td>
<td>Two-step PCR</td>
<td>Sørensen et al. (1993)</td>
</tr>
<tr>
<td>ACT flank L1</td>
<td>CCFCACCCGCCTACCCGGGA</td>
<td>Two-step PCR</td>
<td>This study</td>
</tr>
<tr>
<td>ACT flank L2</td>
<td>CACCCACCCGCAGCTTTT</td>
<td>Two-step PCR</td>
<td>This study</td>
</tr>
<tr>
<td>ACT flank R1</td>
<td>CTCGCCGCGCCCTCTGTCGA</td>
<td>Two-step PCR</td>
<td>This study</td>
</tr>
<tr>
<td>ACT flank R2</td>
<td>AACCTATCCACCCAGCAC</td>
<td>Two-step PCR</td>
<td>This study</td>
</tr>
<tr>
<td>Enterob ampG F1</td>
<td>GTGGTGATCAGCTTTGCTT</td>
<td>ampG sequencing</td>
<td>Schmidt et al. (1995)</td>
</tr>
<tr>
<td>Enterob ampG R1</td>
<td>GTATATTCCACGGTCTCGG</td>
<td>ampG sequencing</td>
<td>Schmidt et al. (1995)</td>
</tr>
</tbody>
</table>
cefoxitin disk approximation tests. The primers used were R0, R1, ACT flank L1, ACT flank L2, ACT flank R1 and ACT flank R2 (Table 1). The PCR conditions were 95 °C for 10 s, followed by 45 cycles at 95 °C for 10 s, 45 °C for 30 s and 72 °C for 1 min, and for the second-round PCR were 95 °C for 10 s, followed by 45 cycles of 95 °C for 10 s, 50 °C for 30 s and 72 °C for 1 min.

To investigate the ampG gene, which encodes a transporter of nureopeptides required for AmpC induction and is essential for high-expression of AmpC β-lactamase in E. cloacae, primers Enterob ampF1 and Enterob ampG R1 were used for PCR and nucleotide sequencing (Schmidt et al., 1995).

Genetic localization of the blaACT-1 gene using PFGE and Southern blot hybridization. To confirm the genetic location of the blaACT-1 gene, transconjugation experiments were performed using Escherichia coli J53 (azide resistant). Genomic DNA was prepared in low-melting-point agarose blocks and digested with the endonucleases S1 or I-CeuI (Sigma-Aldrich). DNA fragments were separated using a CHEF-DR II apparatus (Bio-Rad). Southern blot hybridization with an ampC probe was performed using a digoxigenin DNA labeling and detection kit (Roche Diagnostics).

RESULTS

Bacterial strain and antimicrobial susceptibility testing

The strain was initially identified as Enterobacter cloacae with 96% probability using the VITEK2 GN system (bioMérieux). The strain was resistant to ampicillin (≥32 µg ml⁻¹), amoxicillin/clavulanic acid (≥32 µg ml⁻¹), cefalotin (≥64 µg ml⁻¹), cefoxitin (≥64 µg ml⁻¹), cefotaxime (4 µg ml⁻¹), ceftazidime (16 µg ml⁻¹) and aztreonam (32 µg ml⁻¹), but had intermediate resistance or was susceptible to cefepime (≤1 µg ml⁻¹), imipenem (≤1 µg ml⁻¹) and meropenem (≤0.25 µg ml⁻¹).

Phenotypic detection and spectrophotometric assay for ACT-1 induction

The disk approximation test showed no antagonism between the cefoxitin disk and the ceftazidime, cefotaxime and aztreonam disks compared with E. cloacae ATCC 23355 (Fig. 1). To measure the change in ACT-1 enzyme activity following cefoxitin induction, spectrophotometric hydrolysis assays were repeatedly performed using cephalothin as a substrate. The hydrolysis activity increased from 98.8 to 189.0 mU (mg protein)⁻¹ with cefoxitin induction, thus showing a 1.9-fold increase.

16S rRNA and hsp60 gene sequencing and DNA-DNA relatedness

The best-matching 16S rRNA gene sequence for strain YMC/KN/03/21 was that of E. hormaechei ATCC 49162™ (CCUG 27126™), showing 99.61% (1287/1292) identity. A

Fig. 1. A disk approximation test with cefoxitin (FOX) and cefotaxime (CTX) and aztreonam (ATM). (a) Strain YMC/KN/03/21; (b) E. cloacae ATCC 23355.

Fig. 2. (a) Phylogenetic tree of 16S rRNA gene sequences in the E. cloacae complex. The GenBank accession numbers are: E. hormaechei subsp. hormaechei CIP 103441™, AJ508302; E. ludwigi CIP 108491™, AJ853891; Enterobacter gergoviae JCM 1234™, AB004748; Enterobacter pyrinus KCTC 2520™, AJ010486; Enterobacter cancerogenus LMG 2693™, Z96078; E. asburiae JCM 6051™, AB004744; E. hormaechei subsp. steigerwaltii CIP 108489, AJ853890; E. aerogenes JCM 1235™, AB004750; E. kobei CIP 105566™, AJ508301; Enterobacter amnigenus JCM 1237™, AB004749; E. nimpissuralis LMG 10245™, Z96077; Enterobacter helveticus LMG 23732™, DQ273688; Enterobacter pulvers LMG 6101™, DQ273684; Enterobacter turicensis LMG 23730™, DQ273681; E. cloacae subsp. cloacae ATCC 13047™, AJ147484; E. cloacae subsp. dissolvens LMG 2683™, Z96079; Enterobacter cowanii CIP 107300™, AJ508303; Enterobacter arachidis Ah-143™, EU672801. (b) Phylogenetic tree of hsp60 genes in the E. cloacae complex. The GenBank accession numbers are: E. hormaechei subsp. hormaechei ATCC 49162™, AJ147108; E. hormaechei subsp. steigerwaltii EN 288, AJ543769; E. hormaechei subsp. oharae EN 314, AJ543782; E. cloacae EN 363, AJ543819; E. cloacae EN 316, AJ543874; E. kobe ATCC BAA260™, AJ567899; E. ludwigi EN 119, AJ417114; E. asburiae ATCC 35953™, AJ147141; E. cloacae EN 343, AJ543804; E. nimpissuralis ATCC 9912™, AJ567900; E. cloacae subsp. dissolvens ATCC 23373™, AJ147143; E. cloacae subsp. cloacae ATCC 13047™, EU643113; E. aerogenes JCM 1235™, AB004750; E. gergoviae ATCC 33028™, AJ567897; E. pyrinus ATCC 49851™, AJ567901. Bars indicate nucleotide substitutions per site.
neighbour-joining phylogenetic tree of the 16S rRNA gene sequences showed that the sequence of YMC/KN/03/21 did not cluster with those of the 18 Enterobacter species except for Enterobacter hormaechei subsp. hormaechei ATCC 49162\(^\text{T}\) (CIP 103441\(^\text{T}\); Fig. 2a). The phylogenetic tree of hsp60 sequences showed that YMC/KN/03/21 belonged to a cluster most closely related to E. asburiae ATCC 35953\(^\text{T}\) (CCUG 25714\(^\text{T}\)) (Fig. 2b).
The DNA–DNA similarity of YMC/KN/03/21 with \textit{E.

hormaechei} CCUG 27126$^T$ and \textit{E. asburiae} CCUG 25714$^T$

was 84 and 46\% respectively.

**RT-qPCR to measure mRNA levels of the bla\textsubscript{ACT-1} gene**

To confirm whether expression of the bla\textsubscript{ACT-1} gene was induced by cefoxitin, RT-qPCR was performed. After cefoxitin exposure, the expression levels of the bla\textsubscript{ACT-1} gene increased 1.98 -fold, supporting the result of the cephalothin hydrolysis assay.

**Sequencing of the AmpC \beta-lactamase, ampR and ampG genes**

The nucleotide sequence of the AmpC \beta-lactamase gene showed 100\% identity with bla\textsubscript{ACT-1} of \textit{K. pneumoniae} (GenBank accession no. U58495). Intact ampR genes were found in the upstream region of the bla\textsubscript{ACT-1} gene (Fig. 3a).

The amino acid sequences of AmpR at positions serine 35, arginine 86, glycine 102, aspartic acid 135 and tyrosine 264 were conserved in the index strain (Fig. 3b). A comparison of the internal transcribed spacer promoter regions with those of inducible ACT-1 PABL reported in \textit{K. pneumoniae}.

---

**Fig. 3.** (a) Schematic map of ACT-1 and its flanking regions in YMC/KN/03/21 (3029 bp, GenBank accession no. HQ235645). Open arrows indicate ORFs. (b) Alignment of the amino acid sequences of AmpR of YMC/KN/03/21 and other related strains that produce various AmpC enzymes. Conserved residues are indicated in boxes. Non-identical residues are shaded. (c) The intergenic region of the ACT-1 and ampR genes. The promoter elements and start codons are indicated in boxes. The GenBank accession numbers are: \textit{E. cloacae} GN7471, AB016612; \textit{E. cloacae} subsp. \textit{cloacae} ATCC 13047$^T$, CP001918; \textit{E. cloacae} subsp. \textit{cloacae} NCTC 9394, FP929040; \textit{E. cloacae} ODhyph, AJ278995; \textit{E. cloacae} TR91, DQ478697; \textit{K. pneumoniae} 225, AF362955.
strain 225 (GenBank accession no. AF362955) (Reisbig & Hanson, 2002) is shown in Fig. 3(c).

The amino acid sequence of AmpG of the index strain YMC/KN/03/21 exhibited 98.9 and 98.5 % identity to those of E. cloacae subsp. cloacae ATCC 13047T (GenBank accession no. CP001918) and E. cloacae strain 55 (GenBank accession no. AB194784), respectively. The sequence downstream of the \( \text{bla} \text{ACT-1} \) gene (817 bp) showed the highest identity of 87.1 % with E. cloacae subsp. cloacae ATCC 13047T.

Genetic localization of the \( \text{bla} \text{ACT-1} \) gene using PFGE and Southern blot hybridization

In several conjugation experiments, the \( \text{bla} \text{ACT-1} \) gene was not transferred to Escherichia coli J53 (azide resistant). When the S1 or I-Cell nuclease-treated PFGE gels were blotted with the ampC probe, the results clearly showed that the \( \text{bla} \text{ACT-1} \) gene was located on the chromosome of E. hormaechei strain YMC/KN/03/21 (data not shown).

DISCUSSION

Strain YMC/KN/03/21 was initially misidentified as E. cloacae using an automated biochemical identification system because the list of organisms that can be identified by the system included E. cloacae, Enterobacter aerogenes, E. amnigenus, E. asburiae, E. cancerogenus, E. gergoviae and Enterobacter sakazakii, but not E. hormaechei.

We determined the DNA–DNA relatedness, a reference method for taxonomic classification, between strain YMC/KN/03/21 and E. hormaechei CCUG 27126T and E. asburiae CCUG 25714T, because they were the most closely related organisms in the phylogenetic analysis using 16S rRNA and hsp60 gene sequences. E. asburiae has previously been proposed as an ancestor of ACT-1 PABL (Rottman et al., 2002). Strain YMC/KN/03/21 was confirmed as E. hormaechei.

When bacteraemic patients infected with Enterobacter strains are treated with oximino-cephalosporins for which they have tested susceptible, there is a 20 % probability that the drugs may select for a resistant mutant due to derepression of the \( \beta \)-lactamase (Chow et al., 1991).

The standard method to measure the induction of AmpC enzymes is to assay the \( \beta \)-lactamase activity with or without \( \beta \)-lactams as an inducer. Simple and efficient methods such as a disk approximation test can be an alternative. However, induction of AmpC \( \beta \)-lactamase was not observed in disk approximation tests, which could be explained by too low a cefoxitin concentration for maximal induction. In contrast, a slight enhancement was noted between the disks, probably due to the additional inhibitory effect (Fig. 1a).

A twofold increase in the expression level of \( \text{bla} \text{ACT-1} \) using RT-qPCR was not detectable in the disk approximation test, indicating that this assay was not sensitive enough to measure low-level ACT-1 induction. This might be explained by suboptimal cefoxitin concentrations in the disk diffusion assay, and is perhaps also related to low permeability or high efflux in this strain.

Three other gene products, AmpR, AmpG and AmpD, are important for AmpC \( \beta \)-lactamase induction (Reisbig et al., 2003). AmpR is a DNA-binding protein that positively regulates the expression of the \( \text{ampC} \) gene. AmpG allows muropeptides to enter the cytoplasm for cell-wall recycling, causing a conformational change in AmpR and resulting in AmpC induction. In contrast, inactivation of AmpD causes the derepressed hyperproduction of the AmpC enzyme. Therefore, in this study, the \( \text{ampR} \) and \( \text{ampG} \) genes were sequenced.

The amino acid sequences of AmpR at positions serine 35, arginine 86, glycine 102, aspartic acid 135 and tyrosine 264, which are important for AmpC induction, were conserved in the index strain (Hanson & Sanders, 1999). Comparison of the internal transcribed spacer promoter regions with those of K. pneumoniae strain 225 showed that the promoter sequences were also conserved (Reisbig & Hanson, 2002). The nucleotide sequences at positions 452, 803 and 1118 of \( \text{ampG} \), which are essential for induction of chromosomal AmpC \( \beta \)-lactamase (Hanson & Sanders, 1999), were conserved in the index strain, reflecting the complexity of ACT-1 induction in E. hormaechei.

In summary, we have identified an E. hormaechei strain carrying the ancestral AmpC \( \beta \)-lactamase of plasmid-mediated ACT-1 by using PCR, nucleotide sequencing, phylogenetic analysis of 16S rRNA and hsp60 genes and DNA–DNA relatedness studies. It is also interesting that the production of ACT-1 was weakly induced by cefoxitin, even though it had the \( \text{ampR} \) gene in the upstream region. The \( -10 \) and \( -35 \) promoters in the intergenic regions were intact, reflecting the complexity of ACT-1 induction in E. hormaechei.

ACKNOWLEDGEMENTS

The authors thank Hee Jung Lim and Sori Jong at the Research Institute of Bacterial Resistance, Yonsei University College of Medicine (Seoul, South Korea) for their excellent technical assistance. This research was supported by the Korea Food & Drug Administration in 2006 with grant no. 06042hangnaemae126.

REFERENCES


