PCR detection of *Klebsiella pneumoniae* in infant formula based on 16S–23S internal transcribed spacer

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**A R T I C L E   I N F O**

Article history:
Received 22 January 2008
Received in revised form 6 March 2008
Accepted 6 March 2008

Keywords:
*Klebsiella pneumoniae*
Detection
Infant formula
ITS
PCR
Enrichment

**A B S T R A C T**

A PCR detection based on 16S–23S rDNA internal transcribed spacer (ITS) of *Klebsiella pneumoniae* was developed in the present study. Nineteen different ITS sequences were amplified from 6 strains of *K. pneumoniae* by universal primers. By sequencing and alignment of these sequences to the other homologous in GenBank, species-specific primers of *K. pneumoniae*, Pf/Pr1 and Pf/Pr2, were designed for amplification of the ITS sequence from the operon containing tDNAAla and tDNAIle. Ten type strains and 21 isolates of *K. pneumoniae* were positive to the PCR detection, and all of the non-*K. pneumoniae* reference strains (79 strains) were negative. The enrichment was performed in this procedure with a modified growth media to enrich *K. pneumoniae* from 1.5 CFU/100 g infant formula to about 10^5 CFU/ml in 900 ml of the media. Combination of the enrichment, with the PCR assay can detect 1.5 CFU/100 g infant formula of *K. pneumoniae* within 48 h. Furthermore, *K. pneumoniae* strains KPE050803 and KPE 050830 were identified by this method in 63 infant formula samples.

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1. Introduction

*Klebsiella pneumoniae* can cause severe morbidity and mortality especially among the newborn (Coovadia et al., 1992; Bingen, 1993; Casolari et al., 2005). It is well-known as a hospital-infection pathogen (Horan et al., 1988; Schaberg et al., 1991; Jerassy et al., 2006), which can lead to severe diseases such as septicemia, pneumonia, urinary tract, and soft tissue infections. However *K. pneumoniae* and other organisms known to be present in low levels in powdered infant formula are of increasing importance as neonatal pathogens (Thurm and Gericke, 1994; Brett et al., 2005). Consistent with the need to provide safe feeding for all infants, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) convened a meeting on microorganisms in powdered infant formula (FAO/WHO, 2004). *K. pneumoniae* was included in the hazard identification category “B” by FAO and WHO on microorganisms, in powdered infant formula, which are considered to be an emerging public health issue. The organisms in the category “B” include: *Pantoea agglomerans* and *Escherichia vulneris* (both formally known as *Enterobacter agglomerans*), *Hafnia alvei*, *K. pneumoniae*, *Citrobacter koseri*, *C. freundii*, *Klebsiella oxytoca* and *Enterobacter cloacae* which are well-established causes of illness in infants (e.g. systemic infection, necrotizing enterocolitis and severe diarrhoea) and have been found in low levels in powdered infant formula (Muytjens et al., 1988). Comparison to other *Enterobacteriaceae* in the category “B”, *K. pneumoniae* was the most important pathogen for its severe morbidity and mortality (Vernet et al., 1995). The conventional methods of detection for *K. pneumoniae* were mainly aimed at clinical sample, such as biological specimens and body fluids (Kurupati et al., 2004), and were not suited to the low level pathogen in food sample.

Ribosomal RNA genes have been used to compare taxa in molecular systematics until recent years (Dauga, 2002; Holmes et al., 2004; Janda et al., 2005). However, as the sequences of rRNA gene are highly conserved, the probes and primers chosen for rDNA are less specific to determine closely related species (Fox et al., 1992). Because of less selection pressure, the 16S–23S rDNA internal transcribed spacer (ITS) sequence seems to be more genetically variable and species specific than that of 16S rDNA and 23S rDNA (Gürtler and Stanisich, 1996; Boyer et al., 2001). Primers and probes for the detection of pathogens have been developed in recent years, for example: *Enterobacter sakazakii*, *Salmonella* sp., and *Listeria monocytogenes* (Gravesen et al., 2000; Chiu et al., 2005; Liu et al., 2006).
### Table 1

The bacterial strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae</td>
<td>ATCC 9345, ATCC 10031, ATCC 13883, CMCC 46102, CMCC 46103, CMCC 46104, CMCC 46109, CMCC 46112, CMCC 46113, CP 301, CP 320, CP 368, CP 568, CP 589, CP 981, CP 1131, CP 1266, CP 1508, Nku 91, Nku 238, Nku 333, Nku 897, Nku 1002, Nku 1005, P11, P12, P6, P50 0026, KPE 050803, KPE 050810</td>
</tr>
<tr>
<td>Klebsiella rhinoscleromatis</td>
<td>NCTC 5046, CMCC 46107, CMCC 46111</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>CMCC 53501</td>
</tr>
<tr>
<td>Yersinia enteroxolitica</td>
<td>CMCC 53513</td>
</tr>
<tr>
<td>Shigella sonnei</td>
<td>CMCC 51630, CMCC 51632</td>
</tr>
<tr>
<td>Shigella boydii</td>
<td>ATCC 13880, ATCC 14756</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>SEMQ 030812</td>
</tr>
<tr>
<td>Salmonella enteritidis</td>
<td>SEMQ 030812</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>CMCC 46103, CMCC 46104, CMCC 46108, CMCC 46109, CMCC 46112, CMCC 46113, CP 301, CP 320, CP 368, CP 568, CP 589, CP 981, CP 1131, CP 1266, CP 1508, Nku 91, Nku 238, Nku 333, Nku 897, Nku 1002, Nku 1005, P11, P12, P6, P50 0026, KPE 050803, KPE 050810</td>
</tr>
<tr>
<td>Salmonella enteritidis</td>
<td>CMCC 150201, CMCC 150202, CMCC 150203</td>
</tr>
</tbody>
</table>

*American Type Culture Collection.*

In this study, the PCR detection based on the ITS sequence of *K. pneumoniae* was developed in order to detect the *K. pneumoniae* in the food sample.

### 2. Materials and methods

#### 2.1. Bacterial strains, culture media and genomic DNA extraction

Three taxonomy systems of *Klebsiella* genus, individually classified by Cowan, Bascomb, and Orskov, have been used in the pathogen identification (Podschun and Ullmann, 1998). For avoiding confusion of the strains, the *K. pneumoniae* mentioned in this study only referred to the *K. pneumoniae* in Cowan and Bascomb’s classification and the *K. pneumoniae* subsp. *pneumoniae* in Orskov’s classification. *Klebsiella rhinoscleromatis* mentioned in this table only referred to the *K. rhinoscleromatis* in Cowan and Bascomb’s classification and the *K. pneumoniae* subsp. *rhinoscleromatis* in Orskov’s classification.

To amplify *K. pneumoniae*-specific sequence, alignment of a BLAST in NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/) with IA-ITS sequence was performed, which was shown in Fig. 1. Two pairs of *K. pneumoniae*-specific primers (5′-ATT TGA AGA GGT TGG AAC CGA T-3′)/5′-TTT ACT CTT AAG TTT TTT TTT TCT TGT GCC T-3′) and (5′-ATT GTG AAG ATG TTT CAT TAC TGA TTT GC-3′) were assigned. The primers were synthesized by Sangon Biotech Co. (Shanghai, China). PCR detection was performed using these two pairs of primers. Briefly, 2 μl template DNA (about 10 ng) was amplified in a 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1 mM each of the four dNTPs, 2 units Taq polymerase (TaKaRa Biotech., D0965, Dalian, China), 1 μM of each primer and 2 μl of template DNA (about 10 ng) in a total 50 μl. For ITS amplification, PCR was performed as following: after pre-denaturation at 94 °C, thirty cycles of denaturation at 94 °C for 30 s, annealing to 55 °C for 30 s, and extension at 72 °C for 50 s; with a terminal extension step of 72 °C for 5 min in a PTC-2020 DNA engine (MJ Research, Inc., Waltham, USA). The negative control [water, produced by Milli-Q Biocol A10 water purification system (Millipore Co., Milford, MA, USA)] was included in the each PCR experiment. Prior to sequencing, the PCR products were assayed by 1.5% agarose gel electrophoresis. The gel bands containing different ITS sequence (stained by EB) were cut out and purified by DNA purification Kit (Promega, Inc., Madison, USA). Each sequence was confirmed by repeated and reverse sequencing. Sequencing was performed in an Applied Biosystems 3730 Automated DNA Sequencer. Nineteen ITS sequences of 6 strains *K. pneumoniae* were identified and submitted to GenBank (DQ399552–DQ399570).

#### 2.3. PCR detection of *K. pneumoniae*

A pair of universal primers, EUF (5′-GCT GTT GTG GTA ANG TGT GCG CTG-3′) and REV (5′-GGC ATT TCC AGA TGG GCR AAC CC-3′) were used to amplify the complete ITS sequence of the bacterial genomic DNA according to a previous study (Liu et al., 2006). The PCR reaction conditions were buffer contained 10 mM Tris–HCl, 1.18 ml acetic acid, 1.5 mM MgCl₂, 0.1 mM each of the four dNTPs, 2 units Taq polymerase (TaKaRa Biotech., D0965, Dalian, China), 4 μl of template DNA (about 10 ng) in a total 50 μl. For ITS amplification, PCR was performed as following: after pre-denaturation at 94 °C, thirty cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 50 s; with a terminal extension step of 72 °C for 5 min in a PTC-2020 DNA engine (MJ Research, Inc., Waltham, USA). The negative control [water, produced by Milli-Q Biocol A10 water purification system (Millipore Co., Milford, MA, USA)] was included in the each PCR experiment. Prior to sequencing, the PCR products were assayed by 1.5% agarose gel electrophoresis. The gel bands containing different ITS sequence (stained by EB) were cut out and purified by DNA purification Kit (Promega, Inc., Madison, USA). Each sequence was confirmed by repeated and reverse sequencing. Sequencing was performed in an Applied Biosystems 3730 Automated DNA Sequencer. Nineteen ITS sequences of 6 strains *K. pneumoniae* were identified and submitted to GenBank (DQ399552–DQ399570).
The ITS sequences of *K. pneumoniae* were aligned with other ITS sequences in GenBank by a BLAST. The results show that the ITS of the culture from each strain could be divided into three types: tDNAIle, tDNAAla, and tDNAAla + tDNAIle (IA-ITS) respectively (GenBank accession number DQ399552–DQ399570). The location of the tDNA in the ITS sequence of *K. pneumoniae* is shown in Fig. 2. The sequences, which were downstream of tDNAAla and between tDNAIle and tDNAAla in IA-ITS, were found more genetically variable by a BLAST. On the contrary, the N-ITS and G-ITS are not fit for primer selection, because of their conservation in the other strains of *Klebsiella* spp. and *E. aerogenes* by a BLAST (Figs. 3 and 4). So, two pairs of *K. pneumoniae*-specific primers, PF/Pr1 and PF/Pr2, were assigned in these genetically variable sequences. They are also illustrated in Figs. 1 and 2.

### 2.3. Specificity of primers

The specificity of two pairs of primers used in PCR detection of *K. pneumoniae* was evaluated by PCR amplification of the DNA extracted from 31 *K. pneumoniae* strains, and 79 non-*K. pneumoniae* bacterial strains. The PCR amplified DNA from different strains of *K. pneumoniae* was analyzed by agarose gel electrophoresis. The results showed that only one DNA band of 130 bp was produced by PF and Pr1 primer system in all of the tested *K. pneumoniae* strains, and one DNA band of 260 bp was produced by PF and Pr2 system for all of *K. pneumoniae* (see Fig. 5). On the other hand, non-*K. pneumoniae* strains, including the strains closely related Enterobacteriaceae did not show any positive results in both of the primer systems (result not shown). The result demonstrated these two pairs of primers are highly specific for detection of *K. pneumoniae*.

### 2.4. Pre-enrichment of infant formula for detection of *K. pneumoniae*

Initially, 900 ml of peptone buffered water (pH 7.2) (Oxoid, CM0009, Hampshire, UK) or sterile distilled water was mixed with 100 g of infant formula at a temperature of 45 °C in Erlenmeyer flasks, which were shaken by hand until the powder was dissolved. All tests were performed in triplicate (FDA, 2002). After overnight incubation at 36 °C for 12 h, 20 ml of the culture from each flask was added into 90 ml of EE broth (buffered glucose, brilliant green, bile broth; Oxoid, CM0317, Hampshire, UK). After further overnight incubation, 1 ml of the culture was centrifuged at 8000 rpm for 2 min. After washing gently twice with molecular biological saline (pH 7.0). The suspension was transferred into three tubes (sample A, B and C). Ten-fold serial dilutions, to 10^{-9}, were made in sample A and B respectively. Fifty micro liters of each dilution of sample A series was spread on LB agar plate in triplicate. The colonies on the plates were counted after incubating for 48 h at 37 °C. At the same time, 50 μl of sample B serial dilutions, 10^{-4} to 10^{-9} CFU/tube, was inoculated to 100 g infant formula samples which had been tested no contamination. These inoculated infant formula samples were subjected to pre-enrichment and PCR detection. One milliliter sample C was used to extract the genomic DNA according to Wilson (1990). And then ten-fold serial dilutions, also to 10^{-9} dilution, were made in such DNA extractions. These DNA serial dilutions of sample C were used to evaluate the sensitivity of PCR detection directly.

### 3. Results

#### 3.1. Sequence analysis of ITS in *K. pneumoniae* genome

The ITS sequences of *K. pneumoniae* were aligned with other ITS sequences in GenBank by a BLAST. The results show that the ITS sequences of *K. pneumoniae* contain tDNAAla (G-ITS), no tDNA (N-ITS), and tDNAAla + tDNAIle (IA-ITS) respectively (GenBank accession number DQ399552–DQ399570). The location of the tDNA in the ITS sequence of *K. pneumoniae* is shown in Fig. 2. The sequences, which were downstream of tDNAAla and between tDNAIle and tDNAAla in IA-ITS, were found more genetically variable by a BLAST. On the contrary, the N-ITS and G-ITS are not fit for primer selection, because of their conservation in the other strains of *Klebsiella* spp. and *E. aerogenes* by a BLAST (Figs. 3 and 4). So, two pairs of *K. pneumoniae*-specific primers, PF/Pr1 and PF/Pr2, were assigned in these genetically variable sequences. They are also illustrated in Figs. 1 and 2.

#### 3.2. Sensitivity of PCR detection in *K. pneumoniae* pure culture

Sensitivity was determined by analyzing the serial dilutions of the genomic DNA extracted from the type strains of *K. pneumoniae* ATCC 10031. After PCR amplification, 10 μl of each PCR product was subjected to agarose gel electrophoresis. The results showed that the amplified ITS DNA band can always be found in the column of gels with genomic DNA extracted from about 1.2 × 10^3 CFU bacteria in the PCR tube by primers PF/Pr2, and the specific bands can be observed in 1.2 × 10^2 CFU bacteria by primers PF/Pr1 system.

#### 3.3. Sensitivity of PCR detection of *K. pneumoniae* in infant formula with enrichment

Infant formula of five different brands (Enfamil, Mead Johnson, China; S26 Gold, Wyeth, China; Wonder Sun 1, Wonder Sun, China; NAN 1, Nestle, Denmark; MORINAGA NL-33, Morinaga, Japan) in local supermarket was confirmed to be negative for *K. pneumoniae* in culture using the method described by Muytjens (1988) (see Materials and methods in Section 2.4). One hundred grams of sample (random selected band of infant formula) was inoculated in triplicate with cell dilution of *K. pneumoniae* type strain ATCC 10031 (the range of inoculated cell is 1.5 × 10^6 CFU to 1.5 × 10^5 CFU). The inoculated and three non-inoculated samples were incubated as described in Materials and methods (Section 2.4). The DNA was extracted from culture for PCR assays. The 12 inoculated samples were tested positive, while 3 non-inoculated samples were tested negative by this method (see Fig. 6).
Enterobacter aerogenes detected in two positive samples in both methods. The quantities of Klebsiella pneumoniae (see Materials and methods in Section 2.2). Maray l’Etoil, France) and the method according to Muytjens (1988) infant formula had been assayed by VITEK 32 system (BioMérieux, Inc., the pathogen is in the range of the report (0.19 in Muytjens (1988) (13 of 141). And the concentration (CFU/100 g) of K. pneumoniae were 3.6 (KPE050803) CFU and 15.8 (KPE050830) CFU/100 g infant formula respectively, but it took 6 days. The positive ratio of K. pneumoniae in this study (2 of 63) is lower than that reported by Muytjens et al., 1988). The ITS sequences as a part of rrn operon have been used all along, but it is time consuming. The molecular biological detections of K. pneumoniae, such as formal PCR and real-time PCR, have been used to the clinical samples with high level of the pathogen. In our procedures, enrichment and PCR with species-specific primer fit for the low level (1.5 CFU) of K. pneumoniae in infant formula have been applied. Using the present assay, two strains of K. pneumoniae KPE050803 and KPE050830 were detected in infant formula. The result was confirmed by 3.6 CFU of K. pneumoniae KPE050803 and 15.8 CFU of K. pneumoniae KPE050830 per 100 g in the same sample using the method described by Muytjens (1988). All of PCR-based detection of 63 infant formula samples demonstrated high sensitivity and specificity. The procedure of PCR assay can be completed within 48 h. The entire medium used in pre-culture was not special for K. pneumoniae, but universal for all strains of Enterobacteriaceae (Muytjens et al., 1988). The ITS sequences as a part of rrn operon exist in all bacterial genome, and they are easy to amplify by a pair of

4. Discussion

K. pneumoniae used to be known as an important hospital-infection pathogen, and it is recognized as a food-borne pathogen to infant (FAO/WHO, 2004). The traditional detection of this pathogen has been used all along, but it is time consuming. The molecular biological detections of K. pneumoniae, such as formal PCR and real-time PCR, have been used to the clinical samples with high level of the pathogen. In our procedures, enrichment and PCR with species-specific primer fit for the low level (1.5 CFU) of K. pneumoniae in infant formula have been applied. Using the present assay, two strains of K. pneumoniae KPE050803 and KPE050830 were detected in infant formula. The result was confirmed by 3.6 CFU of K. pneumoniae KPE050803 and 15.8 CFU of K. pneumoniae KPE050830 per 100 g in the same sample using the method described by Muytjens (1988). All of PCR-based detection of 63 infant formula samples demonstrated high sensitivity and specificity. The procedure of PCR assay can be completed within 48 h. The entire medium used in pre-culture was not special for K. pneumoniae, but universal for all strains of Enterobacteriaceae (Muytjens et al., 1988). The ITS sequences as a part of rrn operon exist in all bacterial genome, and they are easy to amplify by a pair of

![Fig. 3. Alignment of the nucleotide sequences of the G-ITS. The roman abbreviation indicates the species to which each strain belongs. K.pne, Klebsiella pneumoniae strain PNE 050206; E.aer, Enterobacter aerogenes strain ATCC 13048.](image1)

![Fig. 4. Alignment of the nucleotide sequences of the N-ITS. The roman abbreviation indicates the species to which each strain belongs. K.pne, Klebsiella pneumoniae strain NKU238; E.aer, Enterobacter aerogenes strain LD118.](image2)

![Fig. 5. Detection of K. pneumoniae by PCR with the specific primes (Pf and Pr1) and (Pf and Pr2). The PCR products were analyzed by electrophoresis in 1% agarose gel. Lanes 1–5: A 130 bp DNA fragment was amplified by Pf and Pr1 from ITS of K. pneumoniae. Lane M, molecular weight marker (100-bp ladder); Lane 1, K. pneumoniae ATCC 9345; Lane 2, K. pneumoniae ATCC 10031; Lane 3, K. pneumoniae ATCC 13883; Lane 4, K. pneumoniae NKU 238; Lane 5, K. pneumoniae CKP 981. A 260 bp DNA fragment was amplified by Pf and Pr2 from ITS of K. pneumoniae. Lane 6, K. pneumoniae ATCC 9345; Lane 7, K. pneumoniae ATCC 10031; Lane 8, K. pneumoniae ATCC 13883; Lane 9, K. pneumoniae NKU 238; Lane 10, K. pneumoniae CKP 981.](image3)

![Fig. 6. Detection of K. pneumoniae in infant formula by PCR with the specific primes. The template DNA was extracted from culture (1.5×10^9 CFU of K. pneumoniae ATCC 10031 inoculated in 100 g infant formula samples). The PCR products were analyzed by electrophoresis of 1% agarose gel. Lanes: lane 1, PCR products amplified by Pf/Pr2 in Enfamil Mead Johnson (China) of infant formula inoculation; lane 2, PCR products amplified by Pf/Pr2 from the brand S 26 Gold Wyeth (China); lane 3, positive control, PCR products amplified by Pf/Pr1 from 10^6 CFU of K. pneumoniae ATCC 10031; lane M, molecular weight marker (100-bp ladder); lane 4, positive control 2, PCR products amplified by Pf/Pr2 of 10^5 CFU of K. pneumoniae ATCC 10031; lane 5, negative control of Pf/Pr1(non-template DNA); lane 6, PCR products amplified by Pf/Pr1 from the brand S 26 Gold Wyeth (China); lane 7, PCR products amplified by Pf/Pr1 of extracted DNA from S 26 Gold Wyeth (China); lane 8, negative control of Pf/Pr2(non-template DNA).](image4)
universal primers, which is in conserved sequence of 16S rDNA terminal 3′ and 23S rDNA 5′ terminal. After development of species-specific primers, the PCR assay based on ITS can be extended to other pathogens in category “A” (Clear evidence of causality), “B” (Causality plausible, but not yet demonstrated), and “C” (Causality less plausible or not yet demonstrated) of the hazard identified by FAO/WHO (FAO/WHO, 2004). In the future it may become necessary and possible to detect all pathogens of Enterobacteriaceae in infant formula. Oligonucleotide probes array detection can be developed based on this assay. The universal primers amplified ITS sequence, the method of selection for species-specific oligonucleotide sequence, and the pre-culture medium can be used directly in the future array.

Acknowledgement

This study was supported financially by a project of the Chinese social commonweal research (project No. 2004DIA2J004-1) and a project of the science and technology by Tianjin government (project No. 043182811).

References


