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Isolation and Identification Methods for Oral *Klebsiella pneumoniae* Involved in Onset of Inflammatory Bowel Disease

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Abstract

Purpose: Recently, it was reported that *Klebsiella pneumoniae* is related to the onset of inflammatory bowel disease including the Crohn disease. It was frequently reported that *K. pneumoniae* was detected in human oral cavities. Regrettably, it currently remains unclear whether *K. pneumoniae* is part of the normal oral flora. The aim of this study was to establish the isolation and identification methods for *K. pneumoniae* from human oral cavities, and investigate its transmission pattern. **Methods:** A selective medium, OKPSM, for the isolation of *K. pneumoniae* from oral cavities was developed in this study. Also, PCR primer for the identification and detection at subspecies level of *K. pneumoniae* was designed. **Results:** OKPSM and PCR method using the primers designed in this study were useful for the isolation and identification of *K. pneumoniae* from human oral cavities. *K. pneumoniae* subsp. *pneumoniae* was detected at 10.0% in 30 saliva samples. On the other hand, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* were detected from no sample. Moreover, *K. pneumoniae* subsp. *pneumoniae* isolates from same subject at 0 month and after 3 months showed same genotypes on AP-PCR using OPA-07 primer. **Conclusion:** These results indicated that human oral cavities were not suitable for the habitat of *K. pneumoniae*.

Keywords

Klebsiella pneumoniae, Selective Medium, Oral Cavity, Multiplex PCR

1. Introduction

At present, the genus *Klebsiella* comprises 27 species and 8 subspecies (http://lpsn.dsmz.de/genus/klebsiella). *Klebsiella* species is a ubiquitous Enterobacteriaceae responsible for various human infectious diseases in immunocompromised individuals [1]. Type species of the genus *Klebsiella* is *Klebsiella pneumoniae*. For the high similarity based on DNA-DNA hybridization, *Klebsiella pneumoniae* species is divided into three subspecies: *K. pneumoniae* subsp. *pneumoniae*, that is the most frequently implicated into human diseases, more specifically urinary tract infections and pneumonia, *K. pneumoniae* subsp. *ozaenae* (formerly *Klebsiella ozaenae*), that is recognized as the main cause of a rhinopharynx chronic inflammatory disease named ozena and as a cause of tracheo bronchopathia [2], and *K. pneumoniae* subsp. *rhinoscleromatis* (formerly *Klebsiella rhinoscleromatis*), involved in a chronic granulomatous disease of upper airway respiratory tract named rhinoscleroma [3]. Because *K. pneumoniae* susp. *pneumoniae* is frequently responsible for nosocomial infection cases, many pathophysiological studies had been previously performed using murine models [4].

K. pneumoniae subsp. *pneumoniae* plays an important role in hospital-acquired infections [5]. Moreover, they are a frequent cause of infections in immunocompromised patients [6] [7] and a potential hazard to patients with extensive burns [8]. As potential members of the commensal flora of the human gut, *K. pneumoniae* subsp. *pneumoniae* are most frequently found in human feces. Recently, it was reported that this organism is related to the onset of inflammatory bowel disease including the Crohn disease [9]. The appearance and spread of hyper-virulent *K. pneumoniae* strains have increased the number of people susceptible to infections; also, strains of this organism have become increasingly resistant to antibiotics, making antibiotic therapy more challenging. Several new antimicrobial-resistance genes were discovered in *K. pneumoniae* subsp. *pneumoniae* before spreading to other pathogens; blaKPC, blaOXA-48-like and blaNDM-1 are examples [10]. Molecular epidemiology analyses allow us to determine the global spread of high-risk clones, thus, providing the necessary data to develop strategies to limit the spread of clinically dangerous strains [11].

A reliable method to isolate three *K. pneumoniae* subspecies from various clinical samples would be an important contribution to epidemiological studies concerning nosocomial infections. Conventional isolation techniques, however, have the disadvantage of being rather insensitive for *K. pneumoniae*, due to the abundance of other bacteria in the samples, which mask the presence of smaller numbers of this organism.

Regrettably, it currently remains unclear whether *K. pneumoniae* is part of the normal oral flora. In our previous pilot study, we tried to detect this organism from the oral samples using conventional selective media for *K. pneumoniae* [12] [13]. However, it was impossible to identify this organism accurately, because those media were not able to completely inhibit the growth of oral bacteria whose number was reported to exceed 600 species [14] and inhibited the growths of some *K. pneumoniae* strains. Thus, a suitable selective medium is needed to assess the prevalence of oral *K. pneumoniae* involved in the onset of inflammatory bowel disease including the Crohn disease. The monitoring of *K. pneumoniae* levels at subspecies level in the oral cavities might be useful for the diagnosis and prevention of *K. pneumoniae* infectious disease.

The detection of *K. pneumoniae* at subspecies level in clinical specimens is important, as it may affect the prognosis and patient management, but identification by conventional biochemical methods can be difficult. The accurate identification and enumeration of *K. pneumoniae* at subspecies level are required to determine their role in various systemic diseases. Although conventional biochemical assays are used to identify three *K. pneumoniae* subspecies, they are often imprecise due to the phenotypic variations displayed by these bacteria. Although sequence analysis of several target genes is the most reliable method, it is expensive, laborious, and time-consuming. Thus, a simple and more reliable assay for identifying three *K. pneumoniae* subspecies is required.

The purpose of the present study was to develop selective media for the isolations of three *K. pneumoniae* subspecies from the human oral samples, and a simple and more reliable assay for identifying them, and also to assess the prevalence of these organisms in the oral cavity.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

All bacterial strains used in the present study are listed in **Table 1**. *K. pneumoniae* strains used in the present study were maintained by cultivating them on Bact™ Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). These organisms were cultured at 30˚C overnight under an aerobic condition.

Strains other than *Klebsiella species* were maintained by cultivating them also on BHI agar. These organisms were cultured at 37˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator (NAPCO® Model 5400; Precision Scientific, Chicago, IL, USA).

2.2. Development of New Selective Medium

2.2.1. Evaluation of Base Medium

BHI agar supplemented with 1% yeast extract (BHI-Y), BHI-Y supplemented with 5% sheep blood (BHI-Y blood), and Nutrient agar (NA) were examined as the base medium in the selective medium. Ten-fold dilutions of cultures were

Table 1. Recovery of *K. pneumoniae* and other bacteria on BHI agar and AISM.

Strain	BHI-Y	OKPSM	Recovery, %
	CFU/ml, $\times 10^8$	CFU/ml, $\times 10^8$	
K. pneumoniae subsp. pneumoniae			
JCM 1662	4.5 ± 0.2[a]	4.4 ± 0.3	98.2
JCM 20034	6.9 ± 0.2	6.8 ± 0.3	97.9
JCM 20348	5.6 ± 0.3	5.5 ± 0.2	97.5
JCM 20507	3.3 ± 0.1	3.3 ± 0.2	99.5
K. pneumoniae subsp. ozaenae			
JCM 1663	5.1 ± 0.2	5.0 ± 0.3	97.5
K. pneumoniae subsp. rhinoscleromatis			
JCM 1664	1.3 ± 0.2	1.3 ± 0.3	97.9
Streptococcus oralis			
ATCC 35037	4.2	0	0
Streptococcus salivarius			
ATCC 33397	1.1	0	0
Streptococcus anginosus			
ATCC 17929	3.3	0	0
Streptococcus mutans			
NCTC 10449	4.2	0	0
Actinomyces naeslundii			
ATCC 12104	0.5	0	0
Actinomyces oris			
ATCC 27044	0.4	0	0
Corynebacterium matruchotii			
ATCC 14266	0.6	0	0
Corynebacterium durum			
ATCC 33449	0.7	0	0
Rothia dentocariosa			
JCM 3067	0.3	0	0
Rothia mucilaginosa			
JCM 10910	0.4	0	0
Rothia aeria			
JCM 11412	1.1	0	0
Neisseria sicca			
ATCC 29256	5.5	0	0

[a]Ave ± SD.

made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates on which bacteria, except *Klebsiella* species, were inoculated were cultured at 37˚C for 48 h in an atmosphere of 5% CO_2 in a CO_2 incubator, and the plates on which *Klebsiella* species were inoculated were cultured at 30˚C for 48 h under an aerobic condition. After cultivation, the number of colony-forming units (CFU)/ml was counted.

2.2.2. Susceptibility Tests

Preliminary studies of antibiotic selection were also performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was used for susceptibility testing [15].

2.3. Recovery of *K. pneumoniae* and Other Representative Oral Bacteria

The recoveries of the *K. pneumoniae* reference strains, *Klebsiella* isolates, and other representative oral bacteria were calculated as CFU/ml on selective medium and compared with those on BHI agar for total cultivable bacteria. All bacterial strains used in the present study are listed in **Table 1**.

All bacterial strains, except *K. pneumoniae*, were pre-incubated in BHI broth at 37˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator. *K. pneumoniae* were pre-incubated in BHI broth at 30˚C overnight in an atmosphere of 5% CO_2 in a CO_2 incubator. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates on which bacteria, except *K. pneumoniae*, were inoculated were cultured at 37˚C for 72 h in an atmosphere of 5% CO_2 in a CO_2 incubator, and those on which *K. pneumoniae* were inoculated were cultured at 30˚C for 24 h under an aerobic condition. After cultivation, the number of CFU/ml was counted.

2.4. Clinical Samples

Thirty volunteers (13 men, 17 women; mean age 43 years, range 18 - 65 years) participated in the present study. They had no systemic disease and received no antibiotic therapy for at least 3 months. All participants were asked not to brush, rinse, or smoke immediately prior to the assessment and not to eat or drink for at least 2 h beforehand.

Paraffin-stimulated whole saliva samples were collected in a sterile microcentrifuge tube. All samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY, USA), and 0.1 ml of each was diluted and inoculated on BHI-Y and selective medium plates. BHI-Y plates for total cultivable bacteria were cultured at 37˚C for 2 days in an atmosphere of 5% CO_2 in a CO_2 incubator, and selective medium plates for *K. pneumoniae* were cultured at 30˚C for 24 h under an aerobic condition. After cultivation, CFU/ml in each sample was calculated. The present study was conducted in accordance with the principles of the Declaration of Helsinki, and was approved by the

Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC20-017).

2.5. Identification of *K. pneumoniae* Isolated from Clinical Samples

Twenty-four of the approximately 50 colonies that grew on the selective medium plate per subject were randomly isolated and subcultured, and their identity was then confirmed by a PCR analysis. After *K. pneumoniae* was isolated from the *K. pneumoniae*-positive subjects, the isolations of this organism from the same subjects were performed six months later again.

2.6. Design of Species-Specific Primers for Three *K. pneumoniae* Subspecies

The design of species-specific primers for three *K. pneumoniae* subspecies was performed as follows. The 16S rRNA sequences of *K. pneumoniae* subsp. *pneumoniae* (accession no. X87276), *K. pneumoniae* subsp. *ozaenae* (AF130982), and *K. pneumoniae* subsp. *rhinoscleromatis* (Y17657), and the *wzc* gene sequences of *K. pneumoniae* subsp. *pneumoniae* (AB719996), *K. pneumoniae* subsp. *ozaenae* (AB719988), and *K. pneumoniae* subsp. *rhinoscleromatis* (AB719987) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and multiple sequence alignment analyses were performed using the CLUSTAL W program; *i.e.*, the 16S rRNA sequences of four species were aligned and analyzed. Homologies among the primers selected for three *K. pneumoniae* subspecies were confirmed by a BLAST search.

2.7. Development of a Multiplex PCR Method Using Designed Primers

A multiplex PCR method for identifying three *K. pneumoniae* subspecies using the designed primers was developed as follows. Bacterial cells were cultured in a BHI broth overnight, and 1 ml of the sample was then collected in a microcentrifuge tube and resuspended at a density of 1.0 McFarland standard (approximately 10^7 CFU in 1 ml of sterile distilled water). A total of 3.6 µl of the suspension was then used as a PCR template. The detection limit for PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The PCR mixture contained 2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.2 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 3.6 µl of the template in a final volume of 20 µl. PCR was performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a mole-

cular size marker.

2.8. Genotyping by AP-PCR Analysis

Genotyping by AP-PCR Analysis AP-PCR analysis for genotyping of *K. pneumoniae* isolates was performed as follows. Subcultured *K. pneumoniae* isolates were suspended in 1.0 McFarland standard in 100 µl of distilled water, and 7.6 µl of the suspension was used as a template for AP-PCR. AP-PCR was performed as described previously [16]. Briefly, the PCR mixture contained 0.2 µM of OPA-7 primer (5'-GAAACGGGTG-3'), 10 µl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 7.6 µl of the template in a final volume of 20 µl. AP-PCR was carried out in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler). AP-PCR conditions included an initial denaturation step at 98˚C for 2 min, 50˚C for 2 min, and 35˚C for 1 min, followed by 30 cycles consisting of 72˚C for 2.5 min, 92˚C for 1.5 min, and 35˚C for 1 min and final extension period of 72˚C for 5 min. PCR products were analyzed by 2.0% agarose gel electrophoresis and visualized by gel staining with ethidium bromide. A 100-bp DNA ladder was used as a molecular size marker (Takara Biomed).

3. Results

3.1. Development of Selective Medium

3.1.1. Selection of Base Medium

The selection of a base medium for the growth of *K. pneumoniae* was performed. Three *K. pneumoniae* subspecies grew well on NA as same as BHI-Y and BHI-Y blood (data not shown). Because of the low cost, NA was ultimately selected as the base medium.

3.1.2. Susceptibility to Antibiotics

Three *K. pneumoniae* subspecies, *i.e.*, *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, and *K. pneumoniae* subsp. *rhinoscleromatis* was more resistant to bacitracin than oral Gram-positive cocci and rods, such as genera *Streptococcus*, *Actinomyces*, *Corynebacterium*, and *Rothia*. The minimal inhibitory concentration (MIC) of bacitracin for *K. pneumoniae* was more than 1000 µg/ml. *K. pneumoniae* was more resistant to lincomycin than oral Gram-negative rods, such as genera *Aggregatibacter*, *Leptotrichia*, and *Eikenella*. The MIC of lincomycin for *K. pneumoniae* was 10 µg/ml. Oral Gram-negative rods were sensitive to 2 µg/ml of lincomycin. *K. pneumoniae* was more resistant to penicillin than oral Gram-negative cocci, such as genus *Neisseria*. The MIC of penicillin for *K. pneumoniae* was 10 µg/ml. Oral Gram-negative cocci were sensitive to 1 µg/ml of penicillin.

3.1.3. Composition of New Selective Medium

The new selective medium, designated oral *K. pneumoniae* selective medium (OKPSM), was composed of the following (per liter): 35 g of nutrient agar, 10 g

of lactose, 20 mg of bromocresol purple, 300 mg of bacitracin, 2 mg of lincomycin, 1 mg of penicillin, and 7.5 mg of amphotericin B. Antibiotics, *i.e.*, bacitracin, lincomycin, penicillin, and amphotericin B were added after the base medium had been sterilized and cooled to 50˚C.

3.2. Multiplex PCR Method for Identifying *K. pneumoniae*

3.2.1. Primer Design

Six specific primers covering the upstream region of the 16S rRNA and *wzc* gene sequence of three *K. pneumoniae* subspecies was designed in the present study (Figure 1 and Figure 2). The specific forward primers were designated as KPF for *K. pneumoniae* subsp. *pneumoniae*, KOF3 for *K. pneumoniae* subsp. *ozaenae*, and KRF for *K. pneumoniae* subsp. *rhinoscleromatis*, whereas the specific reverse primers were designated as KPR for *K. pneumoniae* subsp. *pneumoniae*, KOR3 for *K. pneumoniae* subsp. *ozaenae*, and KRR for *K. pneumoniae* subsp. *rhinoscleromatis*. The amplicon sizes of *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, and *K. pneumoniae* subsp. *rhinoscleromatis* were 549 bp, 152 bp, and 674 bp, respectively.

3.2.2. Detection Limit

Our multiplex PCR method for identifying and detecting three *K. pneumoniae* subspecies, *i.e.*, *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp.

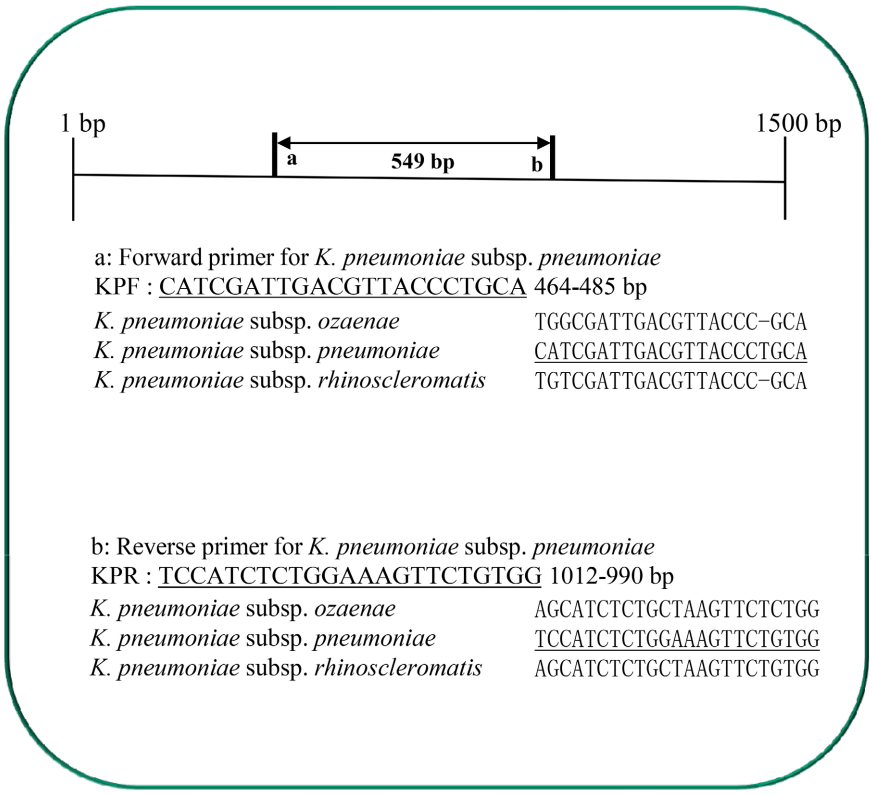


Figure 1. Species-specific primers for 16S rRNA gene of *K. pneumoniae* subsp. *pneumoniae*. The nucleotide sequence of each primer has been underlined.

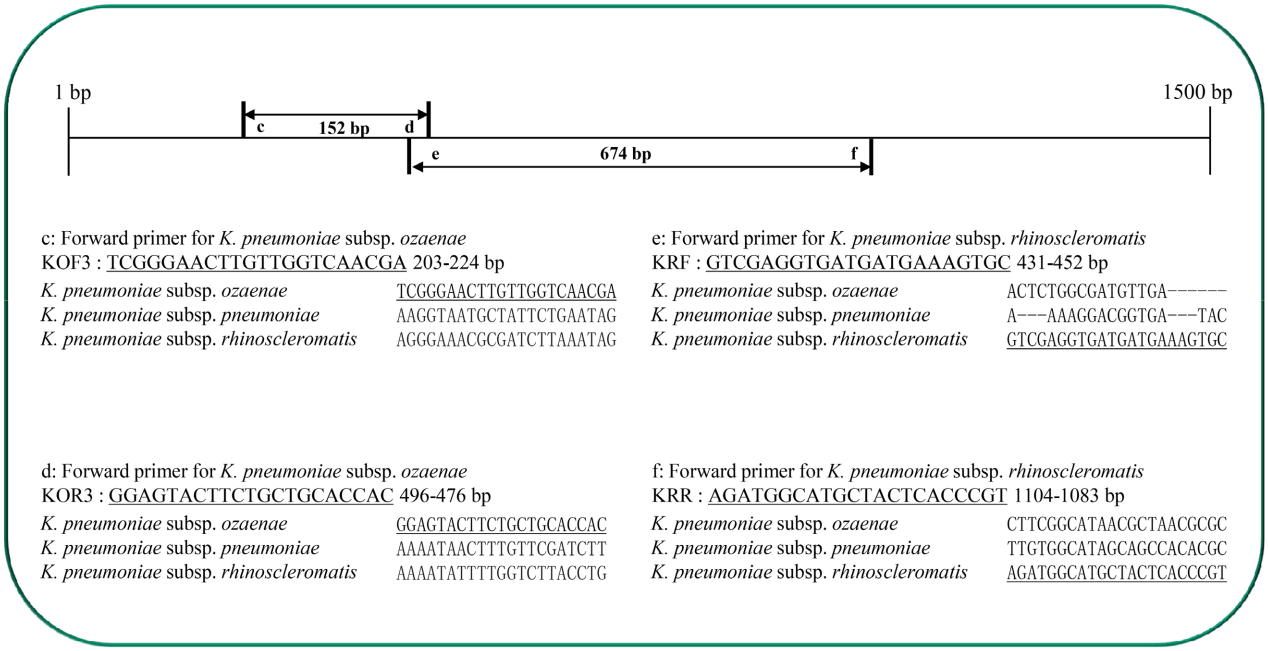


Figure 2. Species-specific primers for the *wzc* gene of *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis*. The nucleotide sequence of each primer has been underlined.

ozaenae and *K. pneumoniae* subsp. *rhinoscleromatis* successfully amplified DNA fragments of the expected size for each species (Figure 3). The detection limit was assessed in the presence of titrated bacterial cells, and the sensitivity of the PCR assay was between 5 × 1 and 5 × 10 CFU per PCR template (5.0 μl) for the *K. pneumoniae* subsp. *pneumoniae*-specific primer set with strain JCM 1662, the *K. pneumoniae* subsp. *ozaenae*-specific primer set with strain JCM 1663, and the *K. pneumoniae* subsp. *rhinoscleromatis*-specific primer set with strain JCM 1664.

3.3. Recovery of *K. pneumoniae* and Inhibition of Other Representative Oral Bacteria on Selective Medium

Table 1 shows the recovery of some *K. pneumoniae* reference strains on OKPSM relative to BHI-Y. The growth recoveries of the *K. pneumoniae* reference strains on OKPSM were between 97.5% and 99.5% (average 98.1%) that on BHI-Y.

Table 1 also shows the inhibition of other representative oral bacteria on OKPSM relative to BHI-Y. The growth of other representative oral bacteria was markedly inhibited on the selective medium.

3.4. Clinical Examination

The detection frequencies of *K. pneumoniae* in saliva samples from thirty healthy subjects are shown in Table 2. *K. pneumoniae* was detected from only three subjects (10.0%). Subspecies of them were *K. pneumoniae* subsp. *pneumoniae*. On the other hand, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* were detected from no one. The mean numbers of this organism in the positive subjects were 7.4×10^2 CFU/ml.

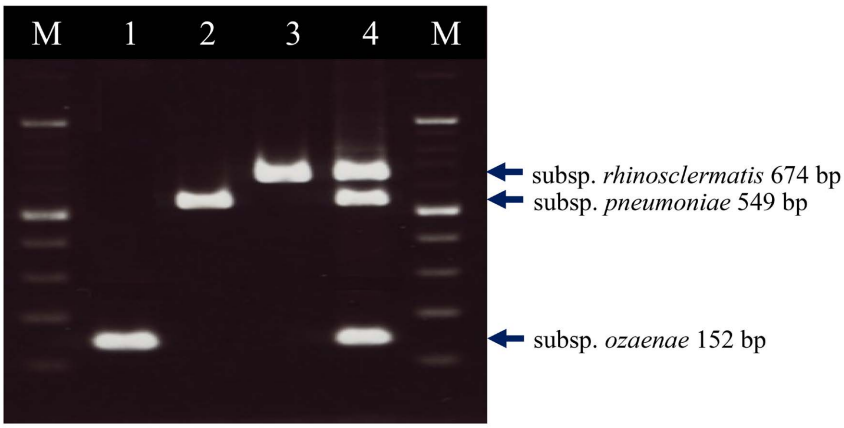


Figure 3. Multiplex PCR for *Klebsiella pneumoniae* subspecies level. Lane: 1: *K. pneumoniae* subsp. *ozaenae* JCM 1663; 2: *K. pneumoniae* subsp. *pneumoniae* JCM 1662; 3: *K. pneumoniae* subsp. *rhinoscleromatis* JCM 1664; 4: Mixture of *K. pneumoniae* subsp. *ozaenae* JCM 1663, *K. pneumoniae* subsp. *pneumoniae* JCM 1662 and *K. pneumoniae* subsp. *rhinoscleromatis* JCM 1664. M, molecular size marker (100 bp DNA ladder).

Table 2. Detection frequencies of *K. pneumoniae* in saliva samples.

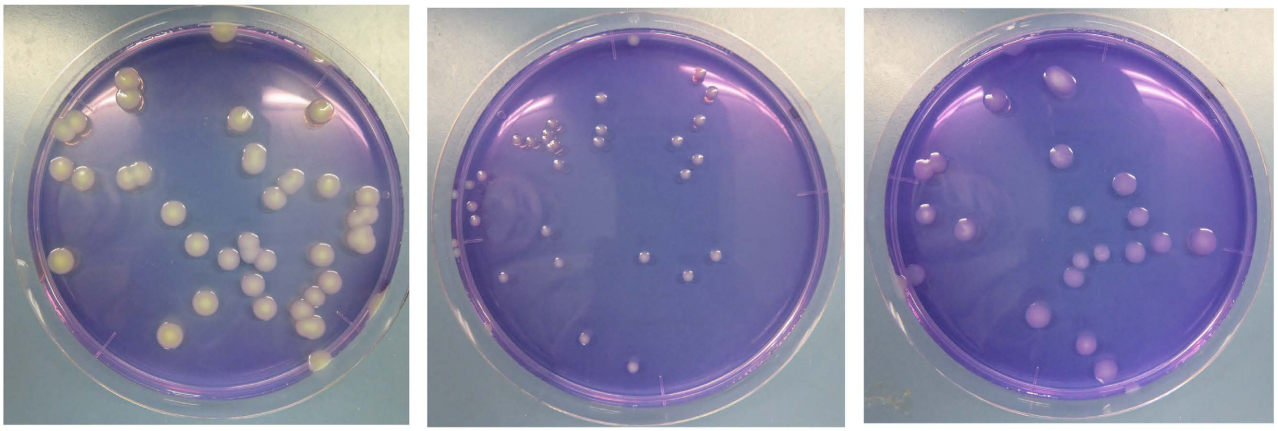
	No. of subjects n = 30 (%, frequency)	No. of *K. pneumoniae* subsp. *pneumoniae* (CFU/ml)	No. of *K. pneumoniae* subsp. *ozaenae* (CFU/ml)	No. of *K. pneumoniae* subsp. *rhinoscleromatis* (CFU/ml)
K. pneumoniae positive	3 (10.0)	7.4×10^2	0	0
K. pneumoniae negative	27 (90.0)	0	0	0

In the first isolation, *K. pneumoniae* colonies on OKPSM commonly had a smooth appearance such as a teardrop.

The colony colors of *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* on OKPSM were light yellow, white and light purple, respectively. Therefore, OKPSM could distinguish three *K. pneumoniae* subspecies by each colony color using differences in acid production from galactose which was added to the medium. The average colony sizes of *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* on OKPSM were 4.6 mm, 2.2 mm and 4.2 mm in diameter, respectively (**Figure 4**).

3.5. Comparison of Genotype of Each *K. pneumoniae* Isolate at 0 Month and after 6 Months

Figure 5 shows the results of genotyping by AP-PCR using OPA-7 primer of each *K. pneumoniae* subsp. *pneumoniae* isolates from subject A, B and C at 0 month and after 6 months. The AP-PCR patterns of *K. pneumoniae* subsp.



K. pneumoniae subsp. *pnumoniae* JCM 1662 | *K. pneumoniae* subsp. *ozaenae* JCM 1663 | *K. pneumoniae* subsp. *rhinoscleromatis* JCM 1664

Figure 4. Colony appearances of three *Klebsiella* subspecies reference strains on OPKSM.

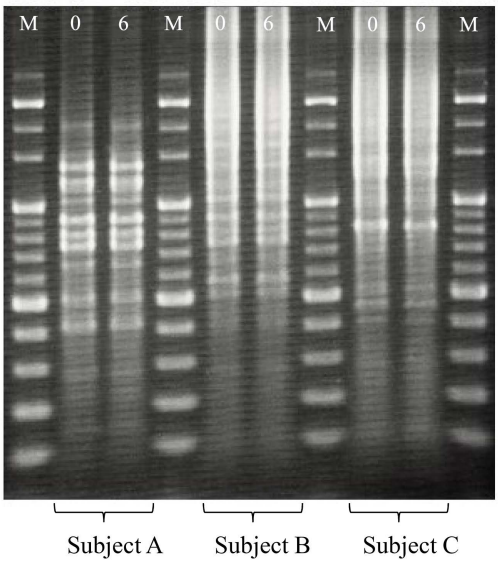


Figure 5. Genetic differences among *K. pneumoniae* subsp. *pneumoniae* isolates from each subject at 0 month and after 6 months with AP-PCR. Lane: 0: *K. pneumoniae* subsp. *pneumoniae* isolates from each subject at 0 month; Lane 6: *K. pneumoniae* subsp. *pneumoniae* isolates after 6 months; Lane M, molecular size maker (100 bp DNA ladder).

pneumoniae that were isolated from each subject at 0 month and after 6 months showed identical genotypes among each individual.

4. Discussion

A reliable selective medium to isolate *K. pneumoniae* from various clinical samples would be an important contribution to epidemiological studies concerning nosocomial infections and antimicrobial resistant strains. Conventional isolation techniques, however, have the disadvantage of being rather insensitive for *Klebsiella* species, due to the abundance of other bacteria in the samples, which mask the presence of smaller numbers of this organism.

In 1970, Thom [12] developed the selective medium for *Klebsiella* species from the human feces, the MacConkey-inositol-carbenicillin (MIC) agar. This medium was based on the MacConkey agar in which lactose was replaced by 1% inositol, with the addition of 100 mg of carbenicillin per liter. This medium owes its elective capacity to the fact that about 97% to 99% of *Klebsiella* species and only 0% to 1% of *E. coli* strains are capable of fermenting inositol and hence appear as red colonies. The selectivity of the medium is due to the presence of carbenicillin to which most *E. coli* strains are susceptible. Resistant *E. coli* strains will appear as pale colonies. Since about 10% to 15% of *Klebsiella* strains are also susceptible to this concentration of carbenicillin, these strains will be missed when this medium is used. Therefore, some investigators reduced the carbenicillin concentration to 10 mg/liter [13] [17].

Cooke *et al.* [13] used MIC agar simultaneously with Simmons citrate agar (SCA), the latter medium allowing the growth of only those types of bacteria that are capable of utilizing citrate as the only carbon source. On SCA, ca. 97% to 99% of *Klebsiella* strains appear as small blue colonies, whereas E. coli is unable to grow on this medium. However, several other types of bacteria are capable of growing on citrate, thus impairing the detection of *Klebsiella* species. So far, however, this method combined with two enrichment media yielded the highest isolation rates of *Klebsiella* species.

Regrettably, it currently remains unclear whether *K. pneumoniae* is part of the normal oral flora. In our previous pilot study, we tried to detect this organism from the oral samples using MIC agar and SCA. However, it was impossible to identify this organism accurately, because those media were not able to completely inhibit the growth of oral bacteria whose number was reported to exceed 600 species [14] and inhibited the growths of some *K. pneumoniae* strains. Thus, a suitable selective medium is needed to assess the prevalence of oral *K. pneumoniae* involved in the onset of inflammatory bowel disease including the Crohn disease.

A useful selective medium for isolating *K. pneumoniae* may contribute to the correct and rapid diagnosis of infectious diseases caused by this organism. However, a selective medium that is useful for the isolation of *K. pneumoniae* from various clinical samples has not ever been developed. In the present study, *K. pneumoniae* strains were more resistant to bacitracin, lincomycin, and penicillin than other representative oral bacteria. The growth of oral bacteria detected in the oral cavity was inhibited by the addition of 300 mg/L bacitracin, 2 mg/L lincomycin, and 1 mg/L penicillin to NA. All of the *K. pneumoniae* reference strains grew well on the new selective medium, designated as OKPSM, while the growth of other bacteria was markedly inhibited (**Table 1**). Moreover, OKPSM allowed for the identification of *K. pneumoniae* by its characteristic colony morphology.

In the present study, we designed species-specific primers with the already mentioned means, for the identification at the subspecies level of *K. pneumoniae* with a PCR method. These primers were able to distinguish *K. pneumoniae* at

the subspecies level and did not display cross-reactivity with each other. Moreover, we developed a multiplex PCR method with the ability to identify and differentiate *K. pneumoniae* at the subspecies level using only each one PCR tubes per sample. Species-specific primers for three subspecies were designed based on the sequences of 16S rRNA and *wzc* gene. Moreover, the PCR method in the present study directly uses bacterial cells with MightyAmp DNA Polymerase Ver.3 (Takara) and is completed within approximately 2 hours.

In this study, *K. pneumoniae* isolates from the same subject at 0 month and after 6 months showed the identical genotype on AP-PCR using OPA-07, respectively. Also, *K. pneumoniae* subsp. *pneumoniae* was detected at only 10.0% in 30 saliva samples. *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* were not detected. The mean numbers of *K. pneumoniae* subsp. *pneumoniae* in those samples were 7.4×10^2 CFU/ml. These results indicated that oral cavity may be only transitory reservoir as *K. pneumoniae* subsp. *pneumoniae* is coming here due to regurgitation or vomiting.

5. Conclusion

We developed a selective medium, designated OKPSM, to isolate *K. pneumoniae* in the oral cavity of humans. Since OKPSM is highly selective for *K. pneumoniae*, it will be useful for assessing the distribution and role of this organism at various locations in humans. The selective medium (OKPSM) and our multiplex PCR method as isolation and identification methods, respectively, for *K. pneumoniae* may contribute to making clear the role of this organism in the aetiology of inflammatory bowel disease including Crohn disease.

Authors' Contributions

Tsuzukibashi O, Fukatsu A, Yamamoto H, Takahashi Y, Usuda K, Fuchigami M, Uchibori S, Komine C, Umezawa K, Hayashi S and Asano T corrected the data. Tsuzukibashi O, Wakami M, Murakami H, Kobayashi T and Fukumoto M drafted and wrote the manuscript. The concept of this manuscript was devised by Tsuzukibashi O. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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