Design of a molecular method for subspecies specific identification of *Klebsiella pneumoniae* by using the 16S ribosomal subunit gene

Nelson Enrique Arenas, MSc[1], Juan Carlos Polanco, PhD[2],
Sandra Milena Coronado, MSc[1], Clara Juliana Durango, QM[1], Arley Gómez, PhD[1,3]

SUMMARY

Introduction: Rhinoscleroma is caused by *Klebsiella pneumoniae* subsp. *rhinoscleromatis* and the ozena infections caused by *K. pneumoniae* subsp. *ozaenae*, both infections affect the upper respiratory tract. In the first clinical phases the symptoms are unspecific, and the disease can be misdiagnosed as a common cold, therefore antimicrobial therapy cannot reach effective results and patients must be following up for several years since the infection became chronic.

Objective: To identify *Klebsiella* subspecies using a specific assay based on amplicons restriction of a gene which encodes 16S subunit ribosomal (*rDNA*16S).

Methodology: Specific restriction patterns were generated; using reported sequences from *rDNA*16S gene and bioinformatics programs MACAW, PFE, GENEDOC and GENE RUNNER. Amplification and restriction assays were standardized.

Results: Predictions *in silico* allowed us to propose an algorithm for *Klebsiella* species and subspecies identification. Two reference strains were included and two clinical isolates which were biotyped and identified by the proposed method. *rDNA*16S gene restriction patterns showed differences regarding the initially identified species for conventional methods. Additionally two patterns of bands were observed for *K. pneumoniae* subsp. *rhinoscleromatis*, indicating the polymorphisms presence in the *rDNA*16S gene.

Conclusions: We confirmed the difficulty to identify *K. pneumoniae* subspecies by conventional methods. Implementation of this technique could allow accurate and rapid differentiation among *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* the aetiological agents of two frequently misdiagnosed infections. Antimicrobial therapy usually could be ineffective, especially in chronic patients. Finally we consider very important to enlarge the study by using more clinical and reference strains.

Keywords: Klebsiella pneumoniae subsp. rhinoscleromatis; Klebsiella pneumoniae subsp. ozaenae; Klebsiella pneumoniae subsp. pneumoniae; Diagnosis.

Diseño de un método molecular para la identificación específica de Klebsiella pneumoniae a nivel de subespecie, usando el gen que codifica para la subunidad ribosomal 16S

RESUMEN

Introducción: El rinoescleroma es causado por *Klebsiella pneumoniae* subsp. *rhinoscleromatis* y la ocena por *K. pneumoniae* subsp. *ozaenae,* respectivamente. Estas infecciones se presentan sobre todo en el tracto respiratorio superior y originan una sintomatología inespecífica en sus fases iniciales por lo cual se pueden confundir con el catarro común. Las dificultades para establecer un diagnóstico oportuno tienen repercusiones negativas en la terapia antimicrobiana, que puede no ser efectiva y hacer que la enfermedad evolucione a una fase crónica cuyo seguimiento en el paciente puede necesitar muchos años.

Objetivo: Diseñar un ensayo molecular para la identificación a nivel de subespecie de bacterias del género *Klebsiella* basado en restricción de amplicones del gen que codifica para la subunidad ribosomal 16S (*ADNr* 16S).

Metodología: Se generaron patrones de restricción específicos, con secuencias informadas del gen *ADNr* 16S y los programas bioinformáticos MACAW, PFE, GENEDOC y GENE RUNNER. Se estandarizaron las condiciones para la amplificación y restricción del ensayo experimental.

Resultados: Las predicciones *in silico* permitieron proponer un algoritmo para identificar a nivel de especie y subespecie los miembros del género *Klebsiella*. Se incluyeron dos cepas de referencia y dos aislamientos clínicos, que fueron biotipificados e identificados por el método propuesto; los patrones de restricción obtenidos del gen *ADNr* 16S evidenciaron diferencias respecto a la especie inicialmente identificada por métodos convencionales. Además, se encontraron dos patrones de bandas en *K. pneumoniae. rhinoscleromatis*, que indican la presencia de polimorfismos en el gen *ADNr* 16S para esta subespecie.

Conclusiones: Se confirmó la dificultad para identificar *K. pneumoniae* a nivel de subespecie por métodos convencionales. La implementación de esta técnica podría permitir la diferenciación temprana entre *K. pneumoniae. ozaenae* y *K. pneumoniae. rhinoscleromatis* que causan dos infecciones tratadas por lo general de forma empírica y como consecuencia de lo anterior, la terapia antimicrobiana suele no ser efectiva, especialmente en enfermos crónicos. Se requiere ampliar los estudios con un número mayor de cepas de referencia y aislamientos clínicos.

Palabras clave: *Klebsiella pneumoniae rhinoscleromatis; K pneumoniae ozaenae; K. pneumoniae pneumoniae; Diagnóstico.*

Historically, taxonomic position of *Klebsiella* genus has been highlighted by several reclassifications and emendations, therefore connoted with controversial framework. Actually recognized *Klebsiella* species include *K. pneumoniae* with three subspecies (*K. pneumoniae* subspecie *pneumoniae*, *K. pneumoniae* subspecie *rhinoscleromatis* and *K. pneumoniae* subespecie *ozaenae*), *K. oxytoca* (with two subgroups), *K. variicola* and also coming *K. granulomatis* (formally named *Calymmatobacterium granulomatis*)[1]. A further classification system recognizes other species as *K. planticola* and *K. terrigena* isolated form environmental sources, *K. ornithinolytica* and *Enterobacter aerogenes,* which has been named as *K. mobilis* and is a genus related specie[2].

Recently these environmental species have been transferred to a new genus called *Raoultella,* which was recognized with basis to differences through meanly by comparative analysis of ribosomal 16S genes (*rDNA 16S*) and the ARN polymerase beta subunit (*RpoB*) from recognized species of *Klebsiella* genus and related enterobacteria, as consequence they constitute two distant lineages on phylogenetic studies[3].

On the other hand, several classification systems based on *Klebsiella* infections has been proposed and thereby international nomenclature has changed and taxon identification could be cumbersome, especially for environmental species[2].

Traditionally laboratories carry out *Klebsiella* differentiation at species and subspecies level by conventional methods; however, these techniques lack of specificity, reproducibility and sometimes there is no correlation when comparison is made with commercial methods such as API 20E system. This situation has been associated with the high similarity and homology on *Klebsiella* genome which make more complex the differentiation with specific genetic markers of clinical isolates and environmental strains[4].

Usually at genus level *K. pneumoniae*, is considered the most clinical important species, since it's responsible of nosocomial and community outbreaks and can generate a wide range of infections due to bacterial ability to colonize and spread out on gastrointestinal, urinary and respiratory tracts[5].

Two *K. pneumoniae* subspecies produce specific respiratory infections. *Klebsiella pneumoniae* subsp. *rhinoescleromatis* is the etiological agent of rhinoscleroma or scleroma infection which is characterized by a granulomatous and chronic process of insidious evolution that affects the mucosa from the upper respiratory tract and might lead to bone invasion and airway obstruction. Clinically, three phases are recognized: catarrhal-atrophic, granulomatous and cicatricial[6].

Klebsiella pneumoniae subsp. *ozaenae* produces ozena or atrophic rhinitis, an infection that compromises the nasal epithelium which results in clinic symptoms such as: anosmia, green mucopurulent and fetid exudates, and nose obstruction whose histological findings are consistent with focal areas of squamous metaplasia, damage of the mucosal glands and therefore mucus secretion alteration[7].

Table 1
Reported sequences of *rDNA* 16S gene for *Klebsiella* species included in this study

Bacterial species	Accession number	Source/Reference
Klebsiella pneumoniae subsp. *pneumoniae*	AF228918	Genbank./ ATCC13886
Klebsiella pneumoniae	Y17656	Genbank /ATCC1383T
Klebsiella pneumoniae	AF228920	Genbank/ NLEP98-0472
Klebsiella pneumoniae	X93214	Genbank/ DSM 30102
Klebsiella pneumoniae	AB004753	Genbank/ JCM1662
Klebsiella pneumoniae subsp. *ozaenae*	AF130982	Genbank/ ATCC:11296
Klebsiella pneumoniae subsp. *ozaenae*	AF228919	Genbank / ATCC13885
Klebsiella pneumoniae subsp. *ozaenae*	Y17654	Genbank/ ATCC11296T
Klebsiella pneumoniae subsp. *rhinoscleromatis*	AF130983	Genbank/ ATCC:13884
Klebsiella pneumoniae subsp. *rhinoscleromatis*	AF009169	Genbank/ NCTC5046
Klebsiella oxytoca	Y17655	Genbank/ ATCC13182T
Klebsiella granulomatis	AF010251	Genbank/ KH 22

Rhinoscleroma and ozena infections are frequently misdiagnosed as a common cold and sinusitis on the initial phases, due to the vague signs and symptoms. These infections show low frequency and commonly the diagnoses are done in a late stage, therapeutic choice is difficult and the results are not fully effective, especially for patients with chronic infection.

The evolution of these infections represents a serious threat for the patient, because of chronic tissue damage and sequelae, recurrences are common events associated in patients infected with resistant strains, which reduce treatment efficacy, increase the economic cost and the patient should be followed up for several years[8].

Available methods for *K. pneumoniae* strains identification allows a much better differentiation when used in combination but are more expensive, laborious and not accurate enough for epidemiological studies because of their low discriminative power and reproducibility[2].

Molecular techniques are a useful tool for infectious diseases diagnosis; the development of polymerase chain reaction (PCR) based systems have been implemented successfully by their reproducibility, accuracy and specificity for specific recognition of minimal differences at genotypic level on highly related organisms and to overcome the conventional test limitations, due to their dependence of physiologic and metabolic activity.

In this study, it was designed a molecular assay based on PRA (PCR Restriction Assay) of *rDNA*16S gene from *K. pneumoniae* for differentiation at the subspecies level.

MATERIALS AND METHODS

Bioinformatic analysis. Reported sequences for the *rDNA* 16S genes of *Klebsiella* genus were obtained from the Genbank and some described sequences for setting phylogenetic relationships of related *Enterobacteriacea* species were also included[9] (Table 1). Multiples alignments were performed with MACAW version 2.0.5; manually edited on PFE software and imported to GENEDOC program for restriction patterns prediction based on *rDNA* 16S sequence alignments. Primers were designed on GENE RUNNER program. Primer specificity and universality evaluation were carried out by using BLASTn program[10].

Phenotypic identification. Two *K. pneumoniae* reference strains (*K. pneumoniae* subsp. *ozaenae* PUJ 040 and *K. pneumoniae* PUJ 017) were obtained from the Microorganism Collection from the Pontificia Universidad Javeriana (recognized by World Federation for Culture Collections) and two clinical isolates (referred as UQ001 y UQ002). MacConkey agar (BD) was used for primary culture. Conventional tests were performed by bacterial culture on: Indole (BD), Simmons Citrate (Oxoid), Voges Proskauer (BD), Triple Sugar-Iron
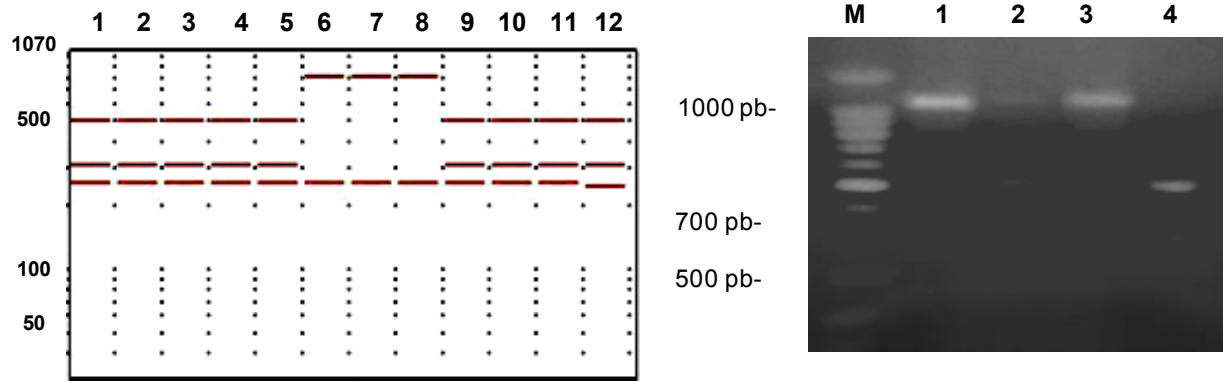
Figure 1. A. *in silico* prediction of restriction patterns with *Ban* II enzyme for *rDNA* 16S Amplicon from *Klebsiella* species. Lines: 1 *Klebsiella pneumoniae* subsp. *pneumoniae*; 2 to 5 *Klebsiella pneumoniae* 6 to 8 *Klebsiella pneumoniae* subsp. *ozaenae;* 9 to 10 *Klebsiella pneumoniae* subsp. *rhinoscleromatis*; 11 *Klebsiella oxytoca*; 12 *Klebsiella granulomatis.* **B.** Gene restriction of *rDNA* gene with *Ban* II enzyme M) Molecular weight marker 100 bp (Promega) 1) *Klebsiella pneumoniae* subsp. *pneumoniae* strain PUJ 017 2) *Klebsiella pneumoniae* subsp. *ozaenae* strain PUJ 040 3-4) *Klebsiella pneumoniae* clinical isolates.

(BD), Urea (BD), Motility, Methyl Red (Oxoid) and gas production (BD) [11]. Reference strains were classified with API 20E commercial system. (BioMèrieux, Marcy l'Etoile, France).

Genotypic methods. DNA genomic extraction was carried out according to standardized protocol for aerobic bacteria[12]. *rDNA 16S* gene amplification from *Klebsiella* genus species were performed under the following reaction conditions: *Klebrib-*1 (5'-GTAATGT CTGGGAAACTGCC-3') [0.5µM] and *Klebrib*-2A (3'-CCACCTTCCTCCAGTTTATC-5') [0.5µM] *Taq* polymerase [0.25U], MgCl [1.5mM], dNTPs [0.2mM], PCR buffer [1X] and DNA sample [10ng/µl], adjusted to a final volume of 50µl. Amplification conditions were 94°C for 1 min; 40 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final step at 72°C for 3 min. PCR product (1069 bp) was purified with Wizard Genomics (DNA Prep., Promega) using the supplier's recommended conditions. Restriction enzyme digestion was performed using *Sal* I, *Ban* II (37ºC) and *Taq* I (65ºC) enzymes, each reaction was performed either with 17 µl *rDNA*, 2 µl Multi Core buffer and 1 µl enzyme restriction (10 U/µl) to a 20 µl final volume. Every solution was mixed and incubated to optimum temperature of each restriction enzyme during 2 hours. Restriction reaction was inactivated with 5 µl EDTA [0.5 M] for 15 minutes. Analysis of the band patterns was obtained by visual interpretation on ethidium bromide stained electrophoresis gels [Agarose 3%].

RESULTS

In silico design of molecular technique. Alignments obtained with reported *rDNA* 16S sequences (Genbank) of *Klebsiella* species showed a high gene similarity interspecifically and intraspecifically. A simple algorithm for specific differentiation at subspecies level with single *rDNA* 16S gene digestions for *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *pneumoniae* was generated. *Ban* II enzyme generated an 816 bp specific fragment for the differentiation of *K. pneumoniae* subsp. *ozaenae* (Figure 1A).

Specific discrimination for *K. pneumoniae* subsp. *rhinoscleromatis* was tested *in silico* through gene pattern prediction of 759 bp and 311 bp of a sequence *rDNA* 16S gene with *Sal* I restriction enzyme (Figure 2A); nevertheless, due to polymorphism described among the *ADNr* 16S gene for *K. pneumoniae* subsp. *rhinoscleromatis*, it was necessary to search an alternative enzyme which allows to differentiate a second gen isotype, thus *Taq* I enzyme generated exclusive band patterns based on polymorphic sequence (Figure 3A). Consequently, it was crucial to take into account this finding for experimental confirmation of predicted band patterns for *K. pneumoniae rhinoscleromatis* reference strains and clinical isolates.

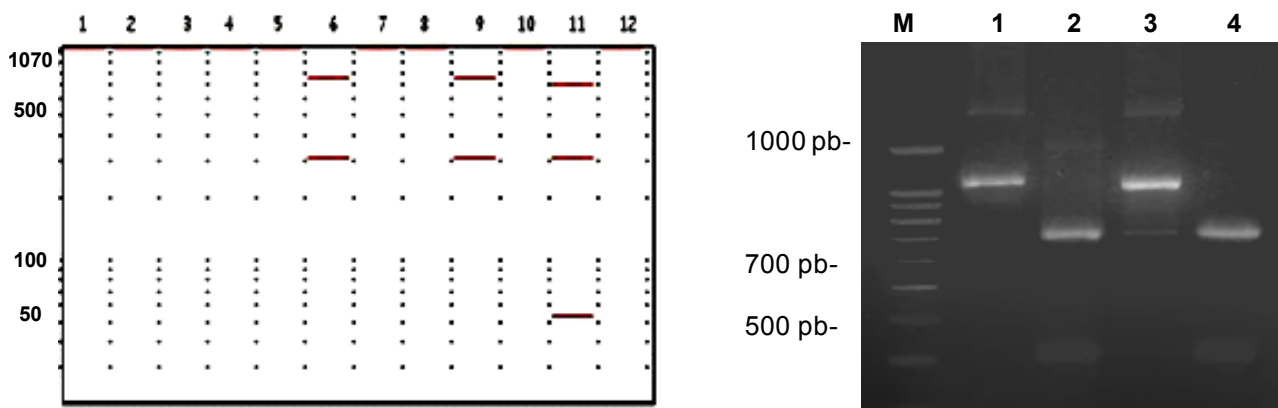Finally, on the basis of clinical significance for differentiation between *K. pneumoniae* and *K. oxytoca,*

Figure 2. A. *in silico* prediction of restriction patterns with *Sal* I for *rDNA* 16S amplicon from *Klebsiella* species. Lines: 1, *Klebsiella pneumoniae* subsp*. pneumoniae*; 2 to 5 *Klebsiella pneumoniae*; 6 to 8 *Klebsiella pneumoniae* subsp. *ozaenae*; 9 to 10 *Klebsiella pneumoniae* subsp. *rhinoscleromatis* 11, *Klebsiella oxytoca*; 12, *Klebsiella granulomatis*. **B.** Gene restriction of *rDNA* 16S with *Sal* I enzyme M) Molecular weight marker 100 bp (Promega) 1) *Klebsiella pneumoniae* subsp. *pneumoniae* PUJ 017 2) *Klebsiella pneumoniae* subsp. *ozaenae* PUJ 040 3-4) *Klebsiella pneumoniae* clinical isolates.
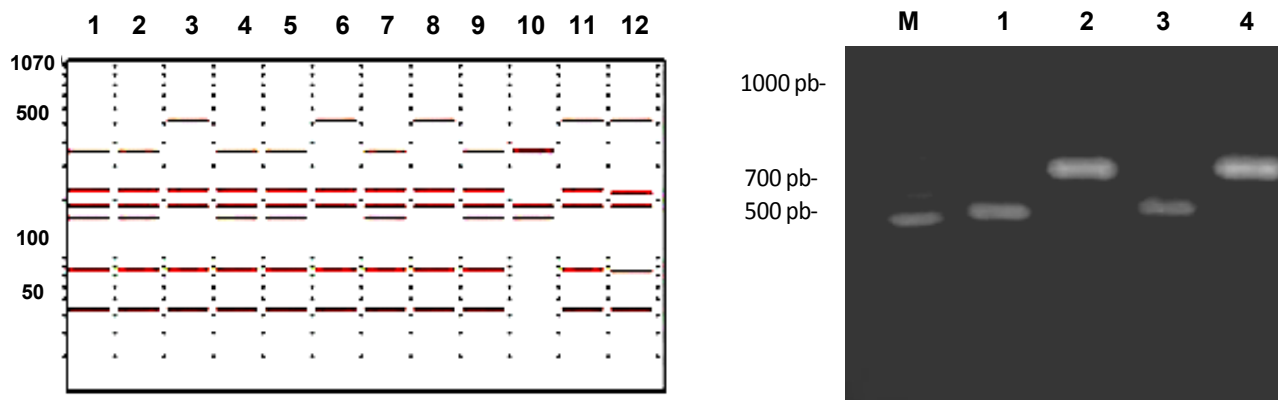

Figure 3. A. *in silico* prediction of restriction patterns with *Taq* I for *rDNA* 16S amplicon from *Klebsiella* species. Lines: 1, *Klebsiella pneumoniae* subsp. *pneumoniae*; 2 to 5 *Klebsiella pneumoniae;* 6 to 8 *Klebsiella pneumoniae* subsp. *ozaenae;* 9 to 10 *Klebsiella pneumoniae* subsp. *rhinoscleromatis*; 11 *Klebsiella oxytoca*; 12, *Klebsiella granulomatis.* **B.** Restriction of *rDNA* 16S with *Taq* I enzyme M) Molecular weight marker 100 bp (Promega) 1) *Klebsiella pneumoniae* subsp. *pneumoniae* PUJ 017; 2) *Klebsiella pneumoniae* subsp. *ozaenae* PUJ 040; 3-4) *Klebsiella pneumoniae* clinical isolates.

additional *in silico* prediction showed that *Rsa* I enzyme can produce 483 bp, 319 bp y 293 bp fragment restriction in comparison with 776 bp and 293 bp fragments predicted in the remaining species (data not shown).

Phenotypic identification of studied strains. The results obtained by using conventional biochemical tests confirmed the significant difficulties for *Klebsiella* differentiation at the species level, even when reference strains were included and biotyping by other methods; some inconsistent findings were obtained for Methyl red and Voges Proskauer reactions.

This result uncertainty did not allow us to define clearly the species identity according to fermentation profile observed on media culture and bacterial strains were characterized only at the genus level. Species confirmation for reference strains were performed by

API 20E commercial system, showing that *K. pneumoniae ozaenae* (strain PUJ 040) generated a numeric pattern concordant with *K. oxytoca*. (Code 5255773), this divergence was based on the differences obtained from biochemical test: indole production, Simmons citrate, urea, Voges Proskauer and sucrose. *K. pneumoniae* (PUJ 017) identification was concordant with *pneumoniae* subspecies (code 1215773) according to supplier's instructions.

Conditions standardization for rDNA 16S amplification and restriction. *ADNr* 16S gene generated an amplification product of 1070 pair bases according to the expected size (Figure 4).

Amplicon restriction with *Ban* II showed the absence of the 816 bp specific fragment for *K. pneumoniae* subsp. *ozaenae* (PUJ 040) even when a reference strain for this subspecies was included; however for the remaining strains a 500 bp, 310 bp y 250 bp pattern bands were observed, which were concordant with *in silico* prediction for *K. pneumoniae* subspecies (Figure 1B). *Sal* I enzyme did not produce any amplicon cuts for *K. rhinoscleromatis* and *K. pneumoniae* subspecies but for *K. ozaenae* subspecies two bands (760 bp and 310 bp approximately) were observed (Figure 2B).

Based on restriction enzyme *Taq* I, similar band patterns (520 bp, 360 bp, 220 bp and 190 bp approximately) were obtained for *K. pneumoniae* subsp. *pneumoniae* (PUJ 017) and for a clinical sample. The remaining strains produced a (740 bp and 220 bp bands) which allowed us to differentiate *K. pneumoniae* subspecies from the other strains. Although, we reported two different patterns obtained from *in silico* prediction only one band pattern for *K. pneumonia* subsp. *rhinoscleromatis* (520, 360, 220 and 190 bp approximately) was experimentally determined (Figure 3B).

The proposed molecular assay starts with *rDNA* 16S amplification on the clinical sample corresponding to *Klebsiella* genus; then an initial restriction with *Ban* II lead to *K.*
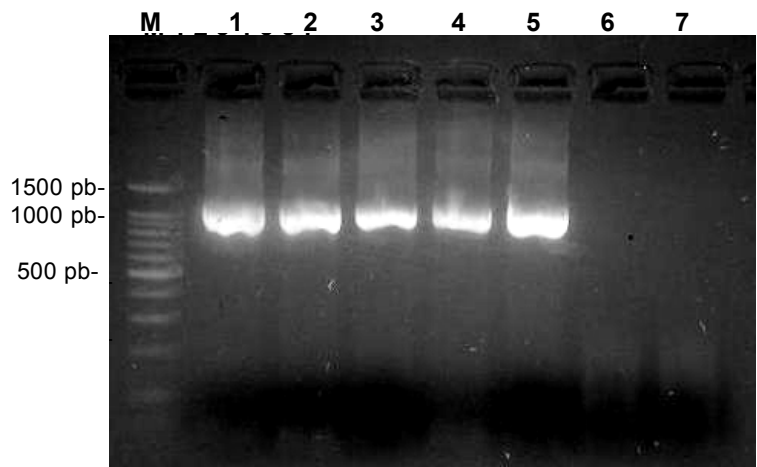

Figure 4. PCR for *rDNA* 16S gene from *Klebsiella* species M) Molecular weight marker 100 bp 1) *Klebsiella pneumoniae pneumoniae* strain PUJ 017; 2) *Klebsiella pneumoniae ozaenae* strain PUJ 040; 3) *Klebsiella oxytoca*; 4) *Klebsiella pneumoniae* subsp. *rhinoscleromatis*-like clinical isolate; 5) *Klebsiella pneumoniae* subsp. *ozaenae* strain PUJ 040; 6) DNA control extraction; 7) Amplification control.

pneumonia subsp. *ozaenae* identification, and afterwards *Sal* I or *Taq* I restriction could differentiate among *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *pneumoniae* (Figure 5).

In general, the proposed molecular assay allows to carry out consecutive digestions to 16S ribosomal gene Amplicon with low cost restriction enzymes commercially available; easy reading and interpretation due to a low bands number (patterns); and none special software is required for data analysis and finally the test implementation would facilitate diagnosis on labs equipped for simple tests of molecular biology.

DISCUSSION

Klebsiella pneumoniae subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenae* typing by conventional tests is based on phenotypic profile assignment which is compared with high concordance of a standard profile; nevertheless, this method can lead to erroneous or incomplete identification due to their inaccuracy for species identification, particularly for subspecies differentiation. For example, biochemical tests such as: Lysine decarboxilase, L-Sorbose acid, L-Proline acid, Simmons Citrate, Dulcitole, Sucrose, Lactose, Inositole, Mucate, Esculine, D-Tartrate decomposition and nitrite to nitrate reduction[11]. Nevertheless, *Klebsiella* species are able to use several carbon resources for their metabolic pathways, which can be detected by automated methods[12,13].

```
┌─────────────────────────────────────────────────┐
│     Clinical isolates and reference strains       │
└─────────────────────────────────────────────────┘
┌─────────────────────────────────────────────────┐
│        Extraction and amplification rDNA          │
└─────────────────────────────────────────────────┘
┌─────────────────────────────────────────────────┐
│               Amplimer restriction                │
└─────────────────────────────────────────────────┘
┌─────────────────────────────────────────────────┐
│              Digestion with Ban II                │
└─────────────────────────────────────────────────┘
```

Klebsiella pneumoniae subsp. ozaenae	Digestion with Sal I o Taq I

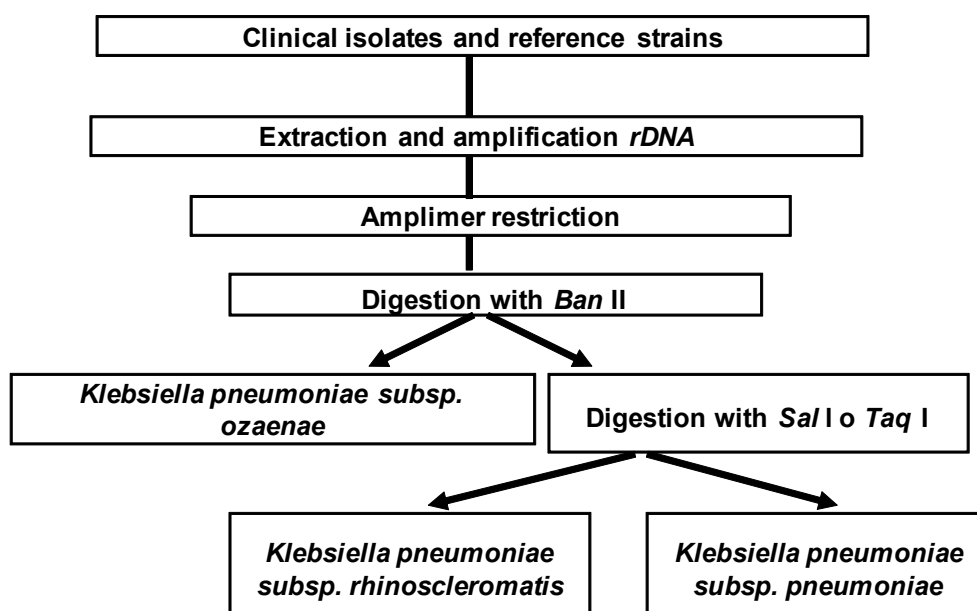| Klebsiella pneumoniae subsp. rhinoscleromatis	Klebsiella pneumoniae subsp. pneumoniae

Figure 5. Algorithm representation for *Klebsiella pneumoniae* subspecie *ozaenae* and *Klebsiella pneumoniae* subspecie *rhinoscleromatis* differentiation by using the PCR Restriction Assay (PRA) of *rDNA* 16S gene.

Hence, the variability of bacterial metabolic behavior justify the design of a new diagnostic tool based on genotyping high throughout molecular techniques, being the analysis of evolutionary conserved and variable genes a useful approach to this diagnostic challenge.

The occurrence of multiple biotypes on *Klebsiella* genus impair the differentiation of these etiologic agents on clinical samples; in fact, bacterial behavior on Red methyl, Urea, Indole production, Citrate utilization tests has been reported as unstable assays since it depend on aerobic conditions, pH, colony differences and variability between systems used for conventional identification[14].

These problems described on commercial systems as API 20E, are influenced by circumstances such as: inoculum size, inoculation time, interpretation technique, and subculture which can affect the reproducibility and as consequence results are discrepant on species identity confirmation[15].

Typing difficulties of *K. pneumoniae* subsp. *rhinoscleromatis* has been recognized not only on traditional methods based on agar and broth cultures, but also on commercial methods as API 20E and recommended panels for *Klebsiella* differentiation[16].

In this paper, it is proposed an algorithm for *K. pneumoniae* subsp. *ozaenae* identification with *Ban* II enzyme, followed by restriction with *Sal* I or *Taq* I for *K. pneumoniae* subsp. *rhinoscleromatis* specific discrimination (Figure 5).

At present, database availability of *rDNA* 16S genes for several bacteria species provides fundamental information for comparative studies by using bio-informatic tools, which can predict accurately gene polymorphism among species correlating with phenotypic features.

Alignments allowed to recognize that *rDNA* 16S gene is highly conserved on *Klebsiella* genus, which is concordant with high similarity values (>99%) reported at intra specific level for this taxonomic group, except for *K. pneumoniae* and *K. oxytoca*. Besides this finding, a phylogenetic relationship for *K. pneumoniae* subspecies in a single branch has been reported[9].

Variability rate among *rDNA* 16S genes for *K. pneumoniae* subsp. *rhinoscleromatis* was found, which could be explained by the differential band patterns identified with *Sal* I and *Taq* I restriction enzymes and the reason can be attributable to the seven *16S ADNr*

gene copies in the genus[1].

Genetic heterogeneity occurrence has been previously described on *K. oxytoca* associated with polymorphism on constitutive genes such as *rDNA* 16S, RNA polymerase subunit-b (RpoB), repetitive entero-bacterial intergenic consensus sequences (ERIC-1R) and b-lactamases encoded chromosomally (OXY-1 y OXY-2), leading to recognition of two genetic subtypes into a same species[17]. Moreover, technical approach to explain intraspecific variation could be not applicable to *DNAr* 16S sequences only for one strain not necessarily derived on taxonomic determination like subspecies or new prokaryotic species[18].

Finally, recent modifications on taxonomy classi-fication of *Klebsiella* genus has sketched out the needs to study the molecular epidemiology, diversity and pathogenesis for *Klebsiella* clinical and environmental isolates because of their differential expression of virulence factors[19].

The proposed method for *K. pneumoniae* discri-mination at subspecies level might generate low band patterns which would help outcomes interpretation.

Restriction enzymes proposed for this molecular assay are commercially available and can be economically favorable in comparison with Amplified Fragment Length Polymorphism (AFLP), Pulsed Field Gel Electropho-resis (PFGE) and Randomly Amplified Polymorphic DNA (RAPD) which can provide low reproducibility and become difficult to interpret[20]; however a need for validation of this technique with reference strains is being here stated out.

Early diagnosis of ozena and rhinoscleroma through a fast and specific molecular method with high discriminatory power at subspecies level might contribute to a rapid therapeutic formulation and thus leading to improvement of patient prognosis, avoiding destructive consequences and sequelae on the respiratory mucosa.

Interest conflict. The authors declare that there are no interest conflicts in this manuscript.

ACKNOWLEDGEMENTS

REFERENCES

1. Martínez J, Martínez L, Rosenblueth M, Silva J, Martínez-Romero E. How are gene sequence analysis modifying bacterial taxonomy? The case of *Klebsiella. Int Microbiol.* 2004; *7:* 261-8.
2. Podschun R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: Epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev.* 1998; *11:* 589-603.
3. Drancourt M, Bollet C, Carta A, Rousselier P. Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. *Int J Syst Evol Microbiol.* 2001; *51:* 925-32.
4. Matsen JM, Spindler JA, Blosser RO. Characterization of *Klebsiella* isolates from natural receiving waters and comparison with human isolates. *Appl Microbiol.* 1974; *28:* 672-8.
5. Sahly H, Podschun R. Clinical, bacteriological, serological aspects of *Klebsiella* infections and their spondylarthropathic sequelae. *Clin Diagn Lab Immunol.* 1997; *4:* 393-9.
6. Andraca R, Edson RS, Kern EB. Rhinoscleroma: A growing concern in the United States? Mayo Clinic Experience. *Mayo Clin Proc.* 1993; *68:* 1151-7.
7. Artiles F, Bordes A, Conde A, Domínguez S, Ramos JL, Suárez S. Rinitis crónica atrófica e infección por *Klebsiella ozaenae. Enferm Infecc Microbiol Clin.* 2000; *18:* 299-300.
8. Maguiña C, Cortés-Escalante J, Oseres-Plengue F, Centeno J, Guerra H, Montoya M, *et al.* Rhinoscleroma: eight Peruvian cases. *Rev Inst Med Trop S Paulo.* 2006; *48:* 295-9.
9. Boye K, Hansen D. Sequencing of 16s rDNA of *Klebsiella*: taxonomic relations within the genus and to other Enterobacteriaceae. *Int J Med Microbiol.* 2003; *292:* 495-503.
10. Stephen AF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997; *25:* 3389-402.
11. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. *Diagnóstico microbiológico. Texto y atlas a color.* 5ª ed. Protocolos 1258-1357. Buenos Aires: Editorial Médica Panamericana; 2001.
12. Tang YW, Ellis NM, Hopkins MK, Smith DH, Dodge DE, Persing DH. Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli. *J Clin Microbiol.* 1998; *36:* 3674-9.
13. Marica D, Kadar I, Rudareanu N, Kadar L, Corlatean M, Miclea I, *et al.* Aerobic conditions and the dynamics of the methyl red reaction in Klebsielleae. *Rev Ig Bacteriol Virusol Parazitol Epidemiol Pneumoftiziol Bacteriol Virusol Parazitol Epidemiol.* 1990; *35:* 71-6.
14. Berlutti F, Thaller MC, Pezzi R. Unusual behaviour of *Klebsiella rhinoscleromatis* strains on API 20E strips. *Microbiology.* 1988; *11:* 77-80.
15. Hansen DS, Aucken HM, Abiola T, Podschum R.

Recommended test panel for diferentiation of *Klebsiella* species on the basis of a trilateral interlaboratory evaluation of 18 biochemical tests. *J Clin Microbiol.* 2004; *42:* 3665-9.

16. Granier SA, Plaisance L, Leflon-Guibout V, Lagier E, Morand S, Goldstein FW, *et al.* Recognition of two genetic groups in the *Klebsiella oxytoca* taxon on the basis of chromosomal beta-lactamase and housekeeping gene sequences as well as ERIC-1 R PCR typing. *Int J Syst Evol Microbiol.* 2003; *53:* 661-8.

17. Clayton RA, Sutton G, Hinkle PS JR, Bult C, Fields C. Intraspecific variation in small-subunit rRNA sequences in genbank: why single sequences may not adequately represent prokaryotic taxa. *Int J Syst Bacteriol.* 1995; *45:* 595-9.

18. Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol.* 2000; *38:* 3623-30.

19. Podschun R, Pietsch S, Holler C, Ullmann U. Incidence of *Klebsiella* species in surface waters and their expression of virulence factors. *Appl Environ Microbiol.* 2001; *67:* 3325-7.

20. Olive DM, Bean P. Principles and applications of methods for DNA-based typing of microbial organisms *J Clin Microbiol.* 1999; *37:* 1661-9.