Differentiation of *Klebsiella pneumoniae* and *K. oxytoca* by Multiplex Polymerase Chain Reaction

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ABSTRACT

To differentiate *Klebsiella* pneumoniae from *K. oxytoca* isolated from clinical samples, a multiplex polymerase chain reaction (PCR) was developed using species specific primers. The position of bands on agarose gel, 108 bp for *K. pneumoniae* and 343 bp for *K. oxytoca*, confirmed strain identity. A total of 331 *Klebsiella* isolates were tested by this method, of which 219 (66%) were identified as *K. pneumoniae* and 52 (16%) as *K. oxytoca*. Isolates, which tested negative with this PCR (n=60) were retested using *Klebsiella* genus specific primers and were confirmed to be *Klebsiella* sp. Results obtained suggest that this newly developed PCR can be used as a diagnostic tool for correct identification and differentiation of *K. pneumoniae* and *K. oxytoca*, two clinically important *Klebsiella* species.

INTRODUCTION

Members of genus *Klebsiella* are opportunistic pathogens that are frequently isolated from various infections in humans and animals.[1] In humans, *Klebsiella* spp in general, and *Klebsiella* pneumoniae and *Klebsiella* oxytoca in particular, are involved in nosocomial infections.[1,2] In animals, *Klebsiella* are mostly associated with sepsis, infections of urinary and respiratory tracts, and mastitis.[3-5] These disease syndromes cause serious economic consequences in some cattle herds.[6] In fact, clinical mastitis due to *Klebsiella* infections results in higher milk losses than those due to *Escherichia coli* and may also result in the death of the affected cows.[7] Both *K. pneumoniae* and *K. oxytoca* are frequently isolated from domestic livestock.

Phenotypic distinction between *K. pneumoniae* and *K. oxytoca* based on existing biochemical tests is not very reliable, and is time consuming and laborious. Current

Table 1. *List of primers used in this study.*

Primer name	Sequence (5'-3')	Target gene	Product size (bp)	Reference
KP (F)	CAA CGG TGT GGT TAC TGA CG	*rpo B*	108	*This study*
(R)	TCT ACG AAG TGG CCG TTT TC			
KO (F)	GAT ACG GAG TAT GCC TTT ACG GTG	*peh X*	343	Kovtunovych *et al.* 2003
(R)	TAG CCT TTA TCA AGC GGA TAC TGG			
Kleb. (F)	CGC GTA CTA TAC GCC ATG AAC GTA	*gyr A*	441	Brisse and Verhoef, 2001
(R)	ACC GTT GAT CAC TTC GGT CAG G			

procedures such as Biolog and API systems often fail to differentiate between species of *Klebsiella*.[8,9] Even the new CHROM Orientation medium does not discriminate properly between *K. oxytoca* and *K. pneumoniae*.[10] These difficulties often lead to mistaken identity between *K. pneumoniae* and *K. oxytoca*.[8,11] To understand the epidemiology of these bacteria and to control their spread, it is imperative to have easy means of discrimination between these two important species of *Klebsiella*. Molecular methods such as polymerase chain reaction (PCR) have been evaluated for the rapid identification of *Klebsiella* in human clinical specimens.[2,11] Kovtunovych et al[11] developed a PCR assay targeting the polygalacturonase gene that could discriminate *K. oxytoca* from other species of *Klebsiella*, and suggested its use for clinical and ecological monitoring of *K. oxytoca*.

Neuberger et al[2] reported the development of a PCR assay that identified genetic sequence of hemolysin gene of *K. pneumoniae* after an initial growth in blood cultures. These last two studies focused primarily on the identification of either *K. pneumoniae* or *K. oxytoca*. In the present study, we have developed a multiplex PCR method to differentiate *K. pneumoniae* from *K. oxytoca* isolated from clinical samples.

MATERIALS AND METHODS

Bacteria

A total of 331 *Klebsiella* isolates were used in this study (Table 1). *Klebsiella* isolates of animal origin (n=104) were obtained from the archives of Minnesota Veterinary Diagnostic Laboratory (MVDL), University of Minnesota, Saint Paul, MN. Using the API 20E identification system (BioMereiux, France), 52 isolates were identified as *K. pneumoniae*, 19 as *K. oxytoca* and 33 as *Klebsiella* sp. In addition, 115 *Klebsiella* isolates were obtained from the Mastitis Laboratory at the College of Veterinary Medicine, St Paul, MN. These isolates were identified simply as *Klebsiella* sp by colony morphology on McConkey agar, motility test, and API 20E identification system (BioMereiux, France). A total of 112 *Klebsiella* isolates from human clinical samples were obtained from the Hennepin County Medical Center, Minneapolis, MN. Using the Vitek 2 (BioMerieux, France) automated identification system and a set of biochemical tests, 107 were identified as *K pneumoniae* and 5 as *K. oxytoca*.

Primers and PCR Amplification

DNA was isolated using PrepMan® reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Species specific primers were used for the amplification of *K. pneumoniae* and *K. oxytoca* in a single reaction mixture (Table 1). PCR was performed using HotStarTaq™ DNA Polymerase kit (Qiagen, Valencia, CA) and the reaction mixture consisted of:

25 μL of master mix, 200 nM of each primer (forward and reverse), 100 ng of template, and water to make final volume of 50 μL. Reaction conditions for PCR were: initial denaturation at 95°C for 15 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 10 min.

***Table 2**. Speciation of Klebsiella isolated from different sources.*

Identification by biochemical test	Sample origin	Identification		
		Number (%) identified as indicated species by PCR		
		K. pneumoniae	***K. oxytoca***	**Untypeable***
K. pneumoniae (n=159)	Animal clinical samples (n=52)	36 (69%)	0	16 (31%)
	Human clinical samples (n=107)	88 (82%)	5 (5%)	14 (13%)
K. oxytoca (n=24)	Animal clinical samples (n=19)	0	17 (89%)	2 (11%)
	Human clinical samples (n=5)	0	4 (80%)	1 (20%)
Klebsiella sp. (n=148)	Animal clinical samples (n=33)	19 (58%)	11 (33%)	3 (9%)
	Mastitis samples (n=115)	76 (66%)	15 (13%)	24 (21%)

**These isolates were tested with genus specific primers and were found to be Klebsiella sp*

The PCR products were visualized on 1.5% agarose gel in Tris-acetate-EDTA buffer by electrophoresis.

A 100 bp DNA ladder was used as molecular weight marker. The position of bands on agarose gel, 108 bp for *K pneumoniae* and 343 bp for *K oxytoca,* confirmed strain identity. PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced both directions with the same primers as used in PCR reactions. Sequences obtained were matched with online database (www.ncbi.nlm.nih.gov) using BLAST. Isolates that tested negative for either *K. pneumoniae* or *K. oxytoca* by the newly developed PCR were retested with *Klebsiella* genus specific primers targeting the gyrA gene (Kleb F and R, Table 1) under the same reaction conditions as for PCR with species specific primers. The PCR products were purified and sequenced.

RESULTS

A total of 331 *Klebsiella* isolates were screened using the newly developed PCR. To confirm primer specificity, PCR was carried out on *Salmonella typhimurium* (ATCC 14028), *E. coli* (ATCC 35248), *Yersinia enterocolitica* (ATCC 9610), *Shigella flexneri* (ATCC 12022), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 13883), and *K. oxytoca* (ATCC 8724). Only *K. pneumoniae* and *K. oxytoca* strains were amplified. Of the 159 *K. pneumoniae* isolates (52 of animal origin and 107 of human origin) screened, PCR identified 124 (78%) as *K. pneumoniae* and 5 (3%) as *K. oxytoca*.

The remaining 30 (19%) isolates could not be identified with either *K. pneumoniae* or *K. oxytoca* specific primers and were classified as untypeable (Table 2). Of the 24 *K. oxytoca* isolates (19 of animal origin and 5 of human origin), 21 (88%) were identified as *K. oxytoca* while the remaining three (12%) tested negative for both *K. pneumoniae* and *K. oxytoca* (Table 2). Of the 148 isolates identified only as *Klebsiella* sp. (33 from animal clinical samples and 115 from mastitis samples), 95 (64%) were identified as *K. pneumoniae* and 26 (18%) as *K. oxytoca* by PCR. The remaining 27 (18%) isolates could not be identified as either *K. pneumoniae* or *K. oxytoca* (Table 2).

All 60 isolates that could not be identified as either *K. pneumoniae* or *K. oxytoca* by this multiplex PCR, were identified as *Klebsiella* sp when tested with genus specific primers. For all these isolates, amplification products of expected size were obtained by PCR and all amplicons were sequenced. Sequence analysis by BLAST did confirm all of these isolates to be *Klebsiella* sp, but they could not be further speciated. All sequences were submitted to the GenBank under accession numbers HM452905-HM452944.

DISCUSSION

For the timely treatment of infections, it is important that diagnostic tests used for the identification of infectious microorganisms are rapid, specific, and sensitive. Members

of the genus *Klebsiella* are opportunistic pathogens which are difficult to identify and are often misclassified in clinical microbiology laboratories.[9,12,13] Current biochemical methods of identification are time consuming and are often inconclusive because related species eg, *K. pneumoniae* and *K. oxytoca* or even *Enterobacter aerogenes*, often present similar biochemical patterns.[14] Correct identification of *Klebsiella* isolates is important for taxonomic and molecular characterization.[12] Molecular methods based on amplification and sequencing of *rpoB, gyrA, parC* genes, and 16S rRNA region have recently become available for the identification of *Klebsiella* sp.[14-16] but there is no PCR based method for the identification/differentiation of *K. pneumoniae* and *K. oxytoca*. This study was designed to fill that gap.

The newly developed multiplex PCR was specific for *K. pneumoniae* and *K. oxytoca* since no amplification was observed with other Gram negative bacteria tested eg, *S. typhimurium, E. coli, Y. enterocolitica,* and *S. flexneri.* The primers used in this study yielded products of 108 bp for *K. pneumoniae* and 343 bp for *K. oxytoca*, enabling clear differentiation of these two species. With this PCR, 78% of the isolates that were identified as *K. pneumoniae* by biochemical tests were identified as *K. pneumoniae*, and 88% of *K. oxytoca* isolates could be confirmed as *K oxytoca*. Thus, 22% of *K. pneumoniae* and 12% of *K. oxytoca* isolates were wrongly identified in biochemical tests. This is not surprising because *R. planticola* and *R. terrigena* are often misdiagnosed as *K. pneumoniae* or *K. oxytoca* by biochemical tests.[9] Others have shown that isolates identified as *K. pneumoniae* by biochemical tests can sometimes be *R. planticola*,[8, 17] *R. terrigena*,[8] or *K. variicola*.[3,18] Similarly, isolates classified as *K. oxytoca* are often *R. planticola*.[8,17] Munoz et al[19] reported that several isolates from mastitis cases that were identified as *K. pneumoniae*, were later identified as *Raoultella* sp. (earlier named as *R. planticola* and *R. terrigena*) upon *rpoB* gene sequencing.

Of the 52 clinical isolates of animal origin which were identified biochemically as *K. pneumoniae*, 69% were confirmed as *K. pneumoniae* by PCR and, 89% of 19 *K. oxytoca* isolates were confirmed as *K. oxytoca*. Of the 107 *K. pneumoniae* isolates from human clinical samples, 82% were identified as *K. pneumoniae* and 80% of five isolates of *K. oxytoca* were correctly identified. This could be because all the isolates used in this study were identified earlier by either biochemical tests or automated identification systems, and these methods are not very accurate for differentiation among *Klebsiella* species.[8] Monnet et al[8] reported that up to 13% of *Klebsiella* isolates are misidentified using biochemical methods. These results further indicate the limitations of the automated identification systems in comparison to PCR. All isolates that could not be differentiated by species-specific primers were tested with Genus-specific primers targeting the gyrA gene of *Klebsiella*. All of these isolates were confirmed to be *Klebsiella* sp but could not be speciated. However, for most of the isolates, BLAST analysis indicated a match with both *K. variicola* and *K. pneumoniae*. This is probably because of very little difference in the sequence of this gene from these two isolates.

Identification of any bacteria up to the species level is very important in clinical microbiology so as to determine the association of a particular species with a disease condition. Though the present study helped in identification and differentiation of only two *Klebsiella* species, further studies are required to differentiate other species of *Klebsiella*. The present study shows that PCR can be a useful tool in diagnostic laboratories for correct identification and differentiation of *K. pneumoniae* and *K. oxytoca* as this method is rapid and specific for these two clinically important *Klebsiella* species.

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