Evaluation of three phenotypic identification systems for clinical isolates of *Raoultella ornithinolytica*

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*Raoultella* spp. have recently been separated from the genus *Klebsiella* based on their molecular characteristics. It was discovered that *Raoultella ornithinolytica* can be misidentified as *Klebsiella oxytoca* by commonly used phenotypic identification systems. Therefore, this study evaluated the ability of three phenotypic systems to identify *R.* ornithinolytica compared with the genotypic methods sequence-specific primer PCR (SSP-PCR), 16S rRNA gene sequence analysis using the MicroSeq 500 system 16S rDNA bacterial identification system or comparison with *GenBank* sequences using BLAST. The phenotypic systems examined in this study were the VITEK 2 GN ID card, the MicroScan Neg Combo 32 panel and API 20E. The SSP-PCR panel was able to distinguish the *R.* ornithinolytica reference strain from other *Raoultella* spp. and *K.* oxytoca. Of the 27 isolates identified as *R.* ornithinolytica by SSP-PCR, VITEK 2 identified all of them as *R.* ornithinolytica. MicroScan and API identified 25 isolates (92.6 %) and 24 isolates (88.9 %) as *K.* oxytoca, respectively. These isolates were ornithine decarboxylase (ODC) negative in all three phenotypic systems. MicroSeq 500 identified 24 isolates (88.9 %) as *R.* ornithinolytica, whereas *GenBank* identification was heterogeneous. Of the 68 isolates identified as *K.* oxytoca by SSP-PCR, 66 isolates (97.1 %) were identified as *K.* oxytoca by VITEK 2, MicroScan and API. MicroScan and API require additional biochemical tests to differentiate between ODC-negative *R.* ornithinolytica and *K.* oxytoca.

INTRODUCTION

*Klebsiella planticola* and *Klebsiella terrigena* were identified in 1981, and *Klebsiella ornithinolytica* was identified in 1989. The first two species are associated primarily with botanical and aquatic environments, whereas the last species, formerly known as ornithine-positive *Klebsiella oxytoca*, was first identified from clinical samples. These species can be discriminated from other *Klebsiella* spp. based on their biochemical reactions, growth temperature, pigment production, G+C content and DNA–DNA hybridization (Bagley et al., 1981; Izard et al., 1981; Sakazaki et al., 1989). For this reason, *K.* planticola, *K.* terrigena and *K.* ornithinolytica were reclassified in the new genus *Raoultella*, based on the 16S rRNA gene and *rpoB* gene sequence analysis of enteric bacteria (Drancourt et al., 2001; Mollet et al., 1997). However, the routine biochemical tests used in the clinical laboratory were not capable of distinguishing clearly between *Raoultella* spp. and *Klebsiella* spp. (Kanki et al., 2002; Monnet & Frenley, 1994; Monnet et al., 1991). Discrimination between the genera *Raoultella* and *Klebsiella* is important to understand their epidemiology and to control their spread in hospitals.

Recently, we found that several clinical isolates identified as *K.* oxytoca by commercial phenotypic systems were subsequently identified as *Raoultella ornithinolytica* using genotypic methods. In this study, we evaluated the abilities of three commercially available phenotypic systems to identify *R.* ornithinolytica compared with the genotypic bacterial identification methods sequence-specific primer PCR (SSP-PCR), which is known to be specific for *R.* ornithinolytica, 16S rRNA gene sequencing using the
MicroSeq 500 system 16S rDNA bacterial identification system or comparison with GenBank sequences (Kanki et al., 2002; Walckenaer et al., 2004).

METHODS

Study design. From March 2006 to March 2007, we collected 114 consecutive clinical isolates that had been identified as *R. ornithinolytica* or *K. oxytoca* using either a VITEK 2 GN ID card or a MicroScan Neg Combo 32 panel at the microbiology laboratories of Seoul National University Bundang Hospital and Seoul National University Hospital (in different provinces of Korea). These isolates were collected from various clinical samples and were received from all wards of the hospitals. We also included the following four reference strains: *R. ornithinolytica* strain ATCC 31898, *Raoultella planticola* strain ATCC 33531, *Raoultella terrigena* strain ATCC 33257 and *K. oxytoca* strain ATCC 700324.

All of the clinical isolates and reference strains were tested and identified using a VITEK 2 GN ID card (bioMérieux), a MicroScan Neg Combo 32 panel (Dade Behring) and an API 20E test kit (bioMérieux), and by SSP-PCR. We used SSP-PCR as the gold standard to identify *R. ornithinolytica* and *K. oxytoca*. For further analysis, we selected the isolates that were identified as *R. ornithinolytica* (n=27) or *K. oxytoca* (n=68) by SSP-PCR, because we were focusing on the misidentification of *R. ornithinolytica* strains by phenotypic systems. The *R. ornithinolytica* isolates identified by SSP-PCR were additionally analysed using 16S rRNA gene sequencing using a MicroSeq 500 system 16S rDNA bacterial identification system (Applied Biosystems) and comparison with GenBank sequences (http://www.ncbi.nlm.nih.gov).

The biochemical tests results of the three phenotypic systems (VITEK 2, MicroScan and API) were analysed to determine the source of the identification discrepancy between the systems. To confirm the results of the phenotypic systems, a conventional ornithine decarboxylase (ODC) test was performed using motility indole ornithine medium (MIO medium; Difco) according to the manufacturer’s recommendation. The incubation period was 5 days as per the guidelines of the Centers for Disease Control and Prevention (Westbrook et al., 2000).

Identification using the three phenotypic systems. API 20E strips were inoculated and processed following the manufacturer’s recommendations and the results were analysed using Apiweb, version 1.2.1. The VITEK 2 GN ID cards were used with a VITEK 2 system, version 4.03. The MicroScan Neg Combo 32 panels were inoculated according to the manufacturer’s recommendations and processed in a MicroScan Walkway 96 apparatus (Dade Behring). The results were analysed using a MicroScan LabPro system, version 1.6.

Identification using SSP-PCR. We used the general model of Granier et al. (2003) (i.e. using SSP-PCR to identify *K. oxytoca*) to design our own panel of β-lactamase (*bla*), histidine decarboxylase (*hdc*) and exo-poly-s-β-galacturonosidase (*pehX*) encoding genes targeted by SSP-PCR to identify *R. ornithinolytica* and *K. oxytoca*. The genes and primers used for the PCR were as follows: the *bla* gene of *R. ornithinolytica* (VO1, 5'-CATACACGCTGAATGGA3'-3', and VO2, 5'-GTTTGTCCGGGGATGT3'-3') (Walckenaer et al., 2004), the *hdc* gene of *R. ornithinolytica* and *R. planticola* (KPE2, 5'-AAAGCTTGGGATATGAC-3', and KPR4, 5'-GTAATGGAAGTTTTTG-3') (Kanki et al., 2002) and the *pehX* gene of *K. oxytoca* (PEH-C, 5'-GATACGGGATGATCCATTGGAC-3', and PEH-D, 5'-TAGCCTTTATCAAGGGGATACGGT-3') (Kovtunovych et al., 2003). A 1121 bp fragment of the *bla* gene, a 724 bp fragment of the *hdc* gene and a 344 bp fragment of the *pehX* gene of the isolates were amplified by PCR using these primers. A suspension of two or three colonies of each isolate was prepared in 500 μl lysis buffer [20 mM Tris/HCl (pH 8.3), 50 mM KCl, 0.1% Tween 20]. The suspension was heat lysed at 94 °C for 10 min and centrifuged at 14,000 g for 5 min. A 200 μl sample of the supernatant of each bacterial extract was stored at −20 °C for amplification. PCRs were performed separately for each of the primer sets. The reaction mixture contents for each PCR were the same. Each 25 μl reaction mixture contained 2.5 mM dNTPs, 10 pmol each PCR primer, 0.6 U Taq DNA polymerase, 2.5 μl 10 × PCR buffer with 15 mM MgCl2 (Takara Bio) and 3 μl DNA template. The PCR conditions for amplification of the *hdc* gene and *pehX* genes were as follows: 3 min of initial denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C, with a final extension step at 72 °C for 5 min. The PCR conditions for amplification of the *bla* gene were the same as the conditions used for the *pehX* gene and *hdc* gene, except that the annealing step was carried out at 50 °C. The amplified DNA was visualized in a 2 % agarose gel containing ethidium bromide (0.5 μg ml−1 final concentration). The clinical isolates were identified by comparing their amplification patterns with those of the reference strains.

Identification by 16S rRNA gene sequencing using MicroSeq 500 and GenBank sequences. A suspension of two or three colonies of each isolate was prepared in 100 μl sterile distilled water. The suspension was heat lysed at 100 °C for 5 min, cooled to room temperature and centrifuged at 14,000 g for 3 min. A 2.5 μl sample of the supernatant of each bacterial extract was used for amplifications. The PCR amplifications were performed in 25 μl volumes, each containing 2.5 mM dNTPs, 10 pmol each PCR primer, 0.6 U Taq DNA polymerase, 2.5 μl 10 × PCR buffer with 15 mM MgCl2 (Takara Bio) and 2.5 μl DNA template. The primers used for amplification were MSQ-F (5'-TGAAGAGTTGTGAATGAC-3') and MSQ-R (5'-ACGCCGCTGTGGGAC-3'). A 527 bp fragment of the 16S rRNA gene of the isolates was amplified from the 5' end of the gene using the following PCR conditions: 10 min of initial denaturation at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C, with a final extension step at 72 °C for 10 min. Positive PCRs and the amplicon size were confirmed using gel electrophoresis. The PCR products were purified using ExoSAP-IT reagent (USB) according to the manufacturer’s instructions prior to sequencing. Forward and reverse sequencing reactions were performed for each amplified product. The sequencing reactions were performed with BigDye terminator reagents on an ABI Prism 3130XL genetic analyzer (Applied Biosystems) using a standard automated sequencer protocol. The sequencing data were analysed using MicroSeq 500, version 2.0, and the GenBank database. The MicroSeq 500 analysis steps were as follows: (i) assembly at the forward and reverse sequences into a consensus sequence, (ii) editing of the consensus sequence to resolve discrepancies between the two strands by evaluation of the electropherograms and (iii) comparison of the consensus sequence in the MicroSeq 500 database. Using the full alignment tool of the MicroSeq 500 software, database comparison generated a list of the closest matches, each with the total number of mismatches. We chose the closest match with the lowest total number of mismatches in the list as the MicroSeq 500 ID of the isolate. The GenBank analysis steps were as follows: (i) all of the sequences were read in the forward and reverse directions and edited as described for MicroSeq 500 sequences, (ii) the sequences were compared with 16S rRNA gene sequences in GenBank using the BLAST program and (iii) for designation to the species or genus level, we used the identification criteria formulated by Drancourt et al. (2000) as a guideline. The detailed criteria were as follows: identification to the species level was defined as a 16S rRNA gene sequence similarity of 99% relative to the prototype strain sequence in GenBank and identification to the genus level was defined as a 16S rRNA gene sequence similarity of 97% relative to the prototype strain sequence in GenBank. Failure
RESULTS

Comparison of microbial identification results from six detection systems

As indicated in Fig. 1, the *R. ornithinolytica* ATCC 31898<sup>T</sup> strain had a profile that was shown by intense bands at 1121 and 724 bp in the *bla* and *hdc* gene PCRs, respectively, whereas no band was observed in the *pehX* gene PCR. *R. planticola* ATCC 33531<sup>T</sup>, *R. terrigena* ATCC 33257<sup>T</sup> and the *Klebsiella pneumoniae* strain showed no band in any of the three PCRs. *K. oxytoca* ATCC 700324 had a profile that was shown by a band at 344 bp present only in the *pehX* gene PCR. Fig. 1 also contains representative examples of clinical isolate profiles. Isolates with the same profile as *K. oxytoca* ATCC 700324 were identified as *K. oxytoca* and those with the same profile as *R. ornithinolytica* ATCC 31898<sup>T</sup> were identified as *R. ornithinolytica* (Fig. 1).

The *R. ornithinolytica* ATCC 31898<sup>T</sup> strain was correctly identified by all three of the phenotypic systems. *R. planticola* ATCC 33531<sup>T</sup> was correctly identified by VITEK 2; however, MicroScan and API incorrectly identified this strain as *K. pneumoniae*. *R. terrigena* ATCC 33257<sup>T</sup> was correctly identified by all three of the phenotypic systems. *R. planticola* ATCC 33531<sup>T</sup> was correctly identified by MicroSeq 500 software also chose *Enterobacter aerogenes*, *R. planticola*, *R. terrigena*, *K. pneumoniae* and *Enterobacter amnigenus* as candidates, each with over 99% matching. However, in all cases, *R. ornithinolytica* was the closest match with the lowest number of mismatches (data not shown). Three other isolates were identified as *E. aerogenes* (n=2) and *Citrobacter koseri* (n=1) by MicroSeq 500. The GenBank database yielded less discriminatory results. GenBank identified nine isolates (33%) as *R. ornithinolytica*, three isolates (11%) as *E. aerogenes* and one isolate (4%) as *C. koseri*. In four isolates (15%), *R. ornithinolytica* and *E. aerogenes* were chosen simultaneously as candidates, each with over 99% matching. They also had the same percentage identity and the same number of mismatches, meaning that there was no way to discriminate between them (data not shown). Ten isolates (37%) were identified as *Klebsiella* spp. at the genus level (Table 1).

Of the 68 isolates identified as *K. oxytoca* by SSP-PCR, 66 isolates (97.1%) were identified as *K. oxytoca* by VITEK 2, MicroScan or API. However, VITEK 2 identified one isolate that was previously identified as *K. oxytoca* by SSP-PCR as *R. ornithinolytica* (Table 1).

Biochemical results of the isolates that were identified as *R. ornithinolytica* by VITEK 2 and as *K. oxytoca* by MicroScan and API

Tables 2, 3 and 4 indicate the expected percentages of positive isolates for *R. ornithinolytica* and *K. oxytoca*, and the results of the individual biochemical tests for the isolates that were identified as *R. ornithinolytica* by VITEK 2 and as *K. oxytoca* by MicroScan and API. Table 2 indicates the results from VITEK 2, Table 3 from MicroScan and Table 4 from API. Unfortunately, the biochemical results of some of the isolates were lost due to technical problems. The same 14 isolates were included in all three of the tables (with an additional 5, 1 and 1 isolates included in Tables 2, 3 and 4, respectively).
Some of the biochemical tests discriminated between \textit{R. ornithinolytica} and \textit{K. oxytoca} in VITEK 2, i.e. l-arabitol, \(\beta\)-\(N\)-acetylgalactosaminidase, \(\alpha\)-tagatose, \(\beta\)-\(N\)-acetyl-galactosaminidase and ODC (Table 2). Of these five biochemical tests, all except ODC were useful to identify \textit{R. ornithinolytica}. For MicroScan and API, ODC was the only discriminatory test between \textit{R. ornithinolytica} and \textit{K. oxytoca}, but the test was not actually useful for the identification of \textit{R. ornithinolytica}. On the contrary, the ODC test led to the misidentification of \textit{R. ornithinolytica} as \textit{K. oxytoca} due to unexpected negativity in the MicroScan and API tests (Tables 3 and 4).

The \textit{R. ornithinolytica} ATCC 31898\(^T\) strain was ODC positive in all three phenotypic systems, whereas the \textit{R. planticola} ATCC 33531\(^T\) and the \textit{K. terrigena} ATCC 33257\(^T\) strains were ODC negative in all three phenotypic systems. The \textit{R. ornithinolytica} ATCC 31898\(^T\) strain also exhibited a positive reaction in a conventional ODC test, whereas \textit{R. planticola} ATCC 33531\(^T\) and \textit{K. terrigena} ATCC 33257\(^T\) showed negative reactions. All 24 isolates that had been identified as \textit{R. ornithinolytica} by VITEK 2 and as \textit{K. oxytoca} by MicroScan and API showed negative reactions in a conventional ODC test (data not shown).

**DISCUSSION**

A reliable phenotypic distinction between \textit{R. ornithinolytica} and \textit{Klebsiella} spp. is a difficult task. VITEK 2 had five discriminatory tests between \textit{R. ornithinolytica} and \textit{K. oxytoca} (see Results). Other than the ODC test, the VITEK 2 tests were useful for the identification of \textit{R. ornithinolytica}. However, MicroScan and API had only one discriminatory test (ODC), and discrimination between \textit{R. ornithinolytica} and \textit{K. oxytoca} was wholly dependent on the result of the ODC test. However, ODC-negative \textit{R. ornithinolytica} isolates have been observed, although they were not clinical isolates but were from animals (Walckenaer et al., 2008). In addition, it has been reported that \textit{E. aerogenes} can be misidentified as \textit{K. pneumoniae} or \textit{K. terrigena} due to a delayed ODC response and poor motility (Ehrhardt et al., 1993). In fact, from the viewpoint of our biochemical results, unexpected ODC negativity was observed in all three of the phenotypic identification systems. Therefore, this dependency of MicroScan and API on the ODC test is considered to be inappropriate to differentiate between \textit{R. ornithinolytica} and \textit{K. oxytoca}.

In this study, we evaluated the capabilities of three commercial phenotype-based systems to identify \textit{R. ornithinolytica} and compared the results with three genotypic identification methods: SSP-PCR and 16S rRNA gene sequence analysis using either MicroSeq 500 or GenBank sequence comparison.

We designed an SSP-PCR panel to target the \textit{bla}, \textit{hdc} and \textit{pehX} genes to identify \textit{R. ornithinolytica} and \textit{K. oxytoca}. It has been suggested that a \textit{bla} gene amplification method could be performed to distinguish \textit{K. oxytoca}, \textit{R. ornithinolytica} and \textit{R. planticola} from each other (Walckenaer et al., 2008). We used \textit{bla} gene primers that were specific to \textit{R. ornithinolytica} in our study. Primers that specifically amplify the \textit{hdc} gene of \textit{R. planticola} and \textit{R. ornithinolytica} have been reported (Kanki et al., 2002), and a specific method to discriminate \textit{K. oxytoca} from other species of the genus \textit{Klebsiella} has been developed based on PCR amplification of the \textit{pehX} gene (Kovtunovych et al., 2003). Using an SSP-PCR panel of the above genes, we were able to successfully distinguish the \textit{R. ornithinolytica} ATCC 31898\(^T\) strain from other \textit{Raoultella} spp., and \textit{K. oxytoca} ATCC 700324 strain from \textit{Raoultella} spp. and \textit{K. pneumoniae}. 

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**Table 1. Discrepancies in microbial identification among the detection systems used in this study**

<table>
<thead>
<tr>
<th>SSP-PCR</th>
<th>VITEK 2 GN ID</th>
<th>MicroScan Neg Combo 32</th>
<th>API 20E</th>
<th>MicroSeq 500</th>
<th>GenBank</th>
<th>% (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RO</td>
<td>RO</td>
<td>RO</td>
<td>RO</td>
<td>RO</td>
<td>Klebsiella spp.</td>
<td>3.7 (1)</td>
</tr>
<tr>
<td>RO</td>
<td>RO</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>RO</td>
<td>RO</td>
<td>29.6 (8)</td>
</tr>
<tr>
<td>RO</td>
<td>RO</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>RO</td>
<td>Klebsiella spp.</td>
<td>25.9 (7)</td>
</tr>
<tr>
<td>RO</td>
<td>RO</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>RO</td>
<td>E. aerogenes / RO</td>
<td>14.8 (4)</td>
</tr>
<tr>
<td>RO</td>
<td>RO</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>RO</td>
<td>E. aerogenes</td>
<td>11.1 (3)</td>
</tr>
<tr>
<td>RO</td>
<td>RO</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>E. aerogenes</td>
<td>Klebsiella spp.</td>
<td>7.4 (2)</td>
</tr>
<tr>
<td>RO</td>
<td>RO</td>
<td>K. oxytoca</td>
<td>C. koseri</td>
<td>C. koseri</td>
<td>C. koseri</td>
<td>3.7 (1)</td>
</tr>
<tr>
<td>RO</td>
<td>RO</td>
<td>K. pneumoniae</td>
<td>K. pneumoniae</td>
<td>RO</td>
<td>RO</td>
<td>3.7 (1)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>RO</td>
<td>E. cloacae</td>
<td>E. cloacae</td>
<td>NA</td>
<td>NA</td>
<td>1.5 (1)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>NA</td>
<td>NA</td>
<td>95.6 (65)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>K. pneumoniae</td>
<td>R. terrigena</td>
<td>NA</td>
<td>NA</td>
<td>1.5 (1)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>K. pneumoniae</td>
<td>K. oxytoca</td>
<td>K. oxytoca</td>
<td>NA</td>
<td>NA</td>
<td>1.5 (1)</td>
</tr>
</tbody>
</table>

NA, Not assayed; RO, \textit{R. ornithinolytica}.

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Table 2. Results of the VITEK 2 GN ID biochemical tests for 19 isolates of those that were identified as *R. ornithinolytica* by VITEK 2 but as *K. oxytoca* by MicroScan and API.

Tests marked in bold are the discriminatory tests between *K. oxytoca* and *R. ornithinolytica*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Expected percentage of positive isolates</th>
<th>Percentage (n) of positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>K. oxytoca</em></td>
<td><em>R. ornithinolytica</em></td>
</tr>
<tr>
<td>Ala-Phe-Pro-arylamidase</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Adonitol</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>L-Pyrolidonyl-arylamidase</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>L-Arabitol</td>
<td>80</td>
<td>1</td>
</tr>
<tr>
<td>Cellobose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>H₂S production</td>
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<td>1</td>
</tr>
<tr>
<td>β-N-Acetylglucosaminidase</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>Glutamyl arylamidase pNA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>γ-Glutamyltransferase</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Fermentation/glucose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Maltose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>D-Mannitol</td>
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<td>99</td>
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<tr>
<td>D-Mannose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>β-Xylosidase</td>
<td>83</td>
<td>99</td>
</tr>
<tr>
<td>β-Alanine arylamidase pNA</td>
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<td>1</td>
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<td>L-Proline arylamidase</td>
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<td>99</td>
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<tr>
<td>Lipase</td>
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<td>1</td>
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<tr>
<td>Palatinose</td>
<td>99</td>
<td>99</td>
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<tr>
<td>Tyrosine arylamidase</td>
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<td>99</td>
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<tr>
<td>Urease</td>
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<td>99</td>
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<tr>
<td>D-Sorbitol</td>
<td>99</td>
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</tr>
<tr>
<td>Sucrose/sucrose</td>
<td>99</td>
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<td>D-Tagatose</td>
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<td>1</td>
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<tr>
<td>Trehalose</td>
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<td>Malonate</td>
<td>98</td>
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<tr>
<td>5-Keto-D-gluconate</td>
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<td>99</td>
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<tr>
<td>L-Lactate alkalinization</td>
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<tr>
<td>α-Glucosidase</td>
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<td>Succinate alkalinization</td>
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<td>99</td>
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<tr>
<td>β-N-Acetylgalosaminidase</td>
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<td>99</td>
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<tr>
<td>Alpha-galactosidase</td>
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<tr>
<td>Phosphatase</td>
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<td>Glycine arylamidase</td>
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<td>ODC</td>
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<tr>
<td>Lysine decarboxylase</td>
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</tr>
<tr>
<td>L-Histidine assimilation</td>
<td>99</td>
<td>75</td>
</tr>
<tr>
<td>Courmarate</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>β-Glucoronidase</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O/129 resistance (vibriostatic compound)</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Glu-Gly-Arg-arylamidase</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L-Malate assimilation</td>
<td>99</td>
<td>86</td>
</tr>
<tr>
<td>Ellman</td>
<td>59</td>
<td>58</td>
</tr>
<tr>
<td>L-Lactate assimilation</td>
<td>94</td>
<td>97</td>
</tr>
</tbody>
</table>

*Values provided by bioMérieux.
One remarkable finding was that all 27 of the isolates that were identified as *R. ornithinolytica* by SSP-PCR were identified as *R. ornithinolytica* by VITEK 2, and as *K. oxytoca* or other species by MicroScan and API. Of the 27 isolates, 24 isolates were also identified as *R. ornithinolytica* using 16S rRNA gene sequence analysis with MicroSeq 500. Furthermore, the other two isolates that were identified as *E. aerogenes* by MicroSeq 500 also showed phenotypes that were closer to *R. ornithinolytica* than to *E. aerogenes* in all three phenotypic systems. For example, these isolates were positive for both the urease and indole tests. In addition, the similarity of the 16S rRNA gene sequences between *R. ornithinolytica* and *E. aerogenes* reaches 99.0% (Boye & Hansen, 2003). These factors indicate that the 27 isolates identified as *R. ornithinolytica* by our SSP-PCR panel are likely to be true *R. ornithinolytica*.

However, for these 27 *R. ornithinolytica* isolates, use of the GenBank database yielded more heterogeneous identification results. Only nine isolates were identified as *R. ornithinolytica*. Four isolates were identified as having multiple candidates with the same percentage identity. Ten isolates failed to be identified to the species level. The reason for GenBank’s heterogeneous identifications for *R. ornithinolytica* isolates, besides the fact that 16S rRNA gene sequences were deposited in the database before the 2001 publication of Drancourt *et al.* (2001), is that the deposited sequences in GenBank are not validated or updated, in contrast to the sequences deposited in MicroSeq 500.

In addition, this study identified a series of ODC-negative *R. ornithinolytica*. Considering the negative conventional ODC tests, the ODC negativity of these isolates appears to be valid and not the result of kinetic problems of the phenotypic systems. In addition, we performed random amplification of polymorphic DNA analysis on five randomly selected isolates to clarify whether these ODC-negative *R. ornithinolytica* isolates were epidemiologically linked (Krawczyk *et al.*, 2003). Fig. 2 shows that the amplification patterns of these five isolates were different from each other. The differences in these patterns indicated that these isolates are not likely to be connected epidemiologically. Additionally, the variations in the clinical situations (i.e. various sources, different infection processes and the distance between the two hospitals; see Methods) did not support the hypothesis that these isolates were epidemiologically linked.

### Table 3. Results of the MicroScan Neg Combo 32 biochemical tests for 15 isolates of those that were identified as *R. ornithinolytica* by VITEK 2 but as *K. oxytoca* by MicroScan and API

Tests marked in bold are the discriminatory tests between *K. oxytoca* and *R. ornithinolytica*.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Expected percentage of positive isolates</th>
<th>Percentage (n) of positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>K. oxytoca</em> <em>R. ornithinolytica</em></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Raffinose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Inositol</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Urea</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td>Lysine</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Tryptophan deaminase</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Citrate</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Colistin (4 µg ml⁻¹) (growth)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sucrose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Adonitol</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>H₂S</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aesculin</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Malonate</td>
<td>99</td>
<td>95</td>
</tr>
<tr>
<td>Cephalotin (8 µg ml⁻¹) (growth)</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td>Arabinose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Melibiose</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Indol</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td><strong>Ornithine</strong></td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>Voges–Proskauer</td>
<td>99</td>
<td>75</td>
</tr>
<tr>
<td>o-Nitrophenyl-β-D-galactopyranoside</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Oxidase</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Values provided by Dade Behring.*
Among the three phenotypic systems, only VITEK 2 was able to identify correctly the ODC-negative *R. ornithinolytica* isolates. However, this greater sensitivity of VITEK 2 was probably achieved at the expense of a lack of specificity, although this effect does not seem to be intentional. In fact, we observed a few isolates that were identified as *R. ornithinolytica* by VITEK 2 but were identified as *R. terrigena*, *Enterobacter* spp., *Escherichia coli*, *C. koseri* and *Kluyvera cryocrescens* by 16S rRNA gene sequencing analysis. However, we excluded these isolates from our analysis because we were focusing on the misidentification of *R. ornithinolytica* strains in the phenotypic systems.

In conclusion, identification of the *Enterobacteriaceae* strains most commonly encountered in a routine clinical microbiology laboratory is usually considered to be straightforward, but identification problems may occur due to diminished or lack of expression of some characteristics, especially in troublesome species in the genera *Klebsiella* and *Raoultella*. Here, we observed a series of ODC-negative *R. ornithinolytica* isolates, causing the commonly used phenotypic identification systems to misidentify these isolates as *K. oxytoca*. Because this study was not an investigation with a wide scope, we cannot make conclusions on the extent of this problem of *R. ornithinolytica* misidentification. However, these misidentifications of *R. ornithinolytica* isolates may be an obstacle to understand their epidemiology. In this study, genotypic identification techniques such as SSP-PCR and 16S rRNA gene sequence analysis with a devoted and updated

### Table 4. Results of the API 20E biochemical tests for 15 isolates of those that were identified as *R. ornithinolytica* by VITEK 2 but as *K. oxytoca* by MicroScan and API

Tests marked in bold are the discriminatory tests between *K. oxytoca* and *R. ornithinolytica*.

<table>
<thead>
<tr>
<th>Substrate</th>
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<th>Percentage (n) of positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>K. oxytoca</em></td>
<td><em>R. ornithinolytica</em></td>
</tr>
<tr>
<td>o-Nitrophenyl-β-D-galactopyranoside</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>80</td>
<td>99</td>
</tr>
<tr>
<td>L-Orynthine</td>
<td>0</td>
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</tr>
<tr>
<td>Trisodium citrate</td>
<td>89</td>
<td>99</td>
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<tr>
<td>H₂S</td>
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<td>0</td>
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<tr>
<td>Urea</td>
<td>78</td>
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<tr>
<td>L-Tryptophan</td>
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<tr>
<td>Indole</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>80</td>
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</tr>
<tr>
<td>Gelatin</td>
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<td>0</td>
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<td>D-Glucose</td>
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<td>D-Mannitol</td>
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</tr>
<tr>
<td>Inositol</td>
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<td>99</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L-Rhamnose</td>
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<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>Melibiose</td>
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<td>100</td>
</tr>
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<td>Amygdalin</td>
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<td>100</td>
</tr>
<tr>
<td>L-Arabinose</td>
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<td>100</td>
</tr>
<tr>
<td>Oxidase</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values provided by bioMérieux.*

Among the three phenotypic systems, only VITEK 2 was able to identify correctly the ODC-negative *R. ornithinolytica* isolates. However, this greater sensitivity of VITEK 2 was probably achieved at the expense of a lack of specificity, although this effect does not seem to be intentional. In fact, we observed a few isolates that were identified as *R. ornithinolytica* by VITEK 2 but were identified as *R. terrigena*, *Enterobacter* spp., *Escherichia coli*, *C. koseri* and *Kluyvera cryocrescens* by 16S rRNA gene sequencing analysis. However, we excluded these isolates from our analysis because we were focusing on the misidentification of *R. ornithinolytica* strains in the phenotypic systems.

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Fig. 2. Random amplification of polymorphic DNA of ODC-negative *R. ornithinolytica* isolates. Lanes: M, 100 bp ladder marker; 1–5, five randomly selected isolates.

---

<table>
<thead>
<tr>
<th>M</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 bp marker</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
database provided better discriminatory power relative to phenotypic techniques. In addition, MicroScan and API required additional biochemical tests to identify the ODC-negative *R. ornithinolytica* isolates from the clinical samples.

**REFERENCES**


