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Incidence and Identification of *Klebsiella planticola* in Clinical Isolates with Emphasis on Newborns

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Studies conducted in France and Germany suggest that up to 19% of clinically identified *Klebsiella* sp. are actually *Klebsiella planticola*, an environmental species that has been attributed to two cases of septicemia, with a rare isolate of *Klebsiella terrigena* (0.4%) being identified. A 1-year survey of newborns on a neonatal ward, also conducted in Germany, reported that 72% of *Klebsiella* sp. were *Klebsiella oxytoca* and 8.7% were *K. planticola*. The tests necessary to identify these species are not found in most clinical identification schemes or in the database matrices of most commercial identification products. To determine the incidence of unrecognized *K. planticola* among the *Klebsiella* sp. isolates in our collection, we used the battery of seven supplemental tests amended from the work of Monnet and Freney to test 352 stock isolates and 84 fresh clinical isolates from four local hospitals. After testing 436 strains of *Klebsiella*, only one strain was identified as a possible *K. planticola* and none was identified as *K. terrigena*. We tested an additional 43 stock strains of *K. oxytoca* isolated from newborns by using eight biochemical tests and found one additional strain of *K. planticola*. The occurrence of *K. planticola* in our collection is far less frequent than that observed in other countries.

Members of the genus *Klebsiella* are responsible for nosocomial infections in adults and for outbreaks in newborn populations in the hospital (3, 7, 9). While these outbreaks are usually caused by *Klebsiella pneumoniae* or *Klebsiella oxytoca*, rare episodes caused by environmental strains have been reported in the literature (6, 11, 15). These environmental strains have also been isolated from sources where the significance of the isolate is unclear (13).

In 1981, Bagley et al. (2) and Izard et al. (10) described two new environmental species, *Klebsiella planticola* and *Klebsiella terrigena*, respectively. These species were differentiated from *K. pneumoniae* and *K. oxytoca* by using tests for temperature-dependent fermentation of glucose, acid production from melezitose and L-sorbose, utilization of carbon sources, and gas production from lactose at 44.5°C (fecal coliform test). Studies from France and Germany suggest that up to 19% of *Klebsiella* spp. identified in clinical settings are actually *K. planticola* (12, 13, 16, 17). *K. planticola* is not routinely identified because clinical identification protocols do not include the tests that are necessary for its identification. Test kits and automated methods have not been able to correctly identify these environmental organisms because they are not included in the identification databases of most diagnostic products nor are substrates included on panels that would differentiate them.

In an attempt to distinguish these environmental species from *K. pneumoniae* and the other clinical *Klebsiella* spp., Monnet and Freney developed a battery of supplemental tests as an adjunct to conventional clinical identification systems (11). These included four carbon substrate assimilation tests (ethanolamine, histamine, D-melezitose, and DL-3-hydroxybutyrate) and two conventional tests (indole production and ornithine decarboxylase).

More recently in Germany, a 1-year survey of newborns on a neonatal ward reported that of all the *Klebsiella* spp. recovered from oropharyngeal and rectal swab specimens, 72% were *K. oxytoca* and 8.7% were *K. planticola* (18).

To identify possible *K. planticola* strains among clinical *Klebsiella* isolates in the United States, and more specifically in newborns, this study consisted of two phases using the supplemental identification schemes described in the literature.

MATERIALS AND METHODS

Bacterial strains. The 395 *Klebsiella* stock isolates taken from the culture collection of the Centers for Disease Control and Prevention (CDC) consisted of *K. pneumoniae* (205 strains), *K. oxytoca* (63 strains), *K. terrigena* (61 strains), *K. planticola* (26 strains), *Klebsiella ornithinolytica* (20 strains), *Klebsiella ozaenae* (10 strains), and *Klebsiella rhinoscleromatis* (10 strains). Most strains of *K. terrigena* were European environmental in origin and most *K. planticola* were from environmental sources in the United States. Of the 63 *K. oxytoca* isolates, 43 were from newborns. Strains from the CDC culture collection were removed from storage at −70°C. Each isolate was passed three times on 5% sheep blood agar plates (BD Biosciences, Sparks, Md.) at 35°C prior to testing. They were identified on conventional media as previously described (5) with some modifications by Hickman and Farmer (8). Incubations were at 35°C, and test results were read at 24 and 48 h and 7 days, unless otherwise noted. Commercial media were used whenever possible. This battery of media does not routinely contain the discriminators needed to distinguish *K. planticola* or *K. terrigena* from *K. pneumoniae* or *K. oxytoca*.

In addition, 84 fresh clinical isolates were randomly collected from four Atlanta area hospital laboratories and were identified as either *K. pneumoniae* (77) or *K. oxytoca* (7) by using commercial identification systems that included MicroScan (20 isolates) (Dade Behring, Inc., West Sacramento, Calif.) and UniScept and Vitek (bioMérieux, Hazelwood, Mo.) (32 isolates each). These isolates were received on Trypticase soy agar slants and were maintained on 5% sheep blood agar plates. They were passed no more than five times prior to testing in these protocols.

These 352 stock strains and 84 clinical isolates were tested by using the method of Monnet and Freney that included indole production, ornithine decarboxylation, and assimilation of ethanolamine, histamine, D-melezitose, and DL-hydroxybutyrate, to which was added acid production from sorbose (Table 1).

The 43 *K. oxytoca* stock strains specifically isolated from newborns were re-identified by using selected supplemental tests to determine the presence of *K. planticola*. The supplemental tests chosen were fecal coliform, fermentation of D-melezitose and sorbose, degradation of pectate, pigmentation on D-gluconate ferric citrate agar, production of indole, fermentation of glucose at 5°C, and utilization of *m*-hydroxybenzoate as the sole carbon source (Table 2).

Carbon source utilization. The carbon substrates tested included ethanolamine, histamine, D-melezitose, and DL-3-hydroxybutyrate (Sigma Chemical Co., St. Louis, Mo.) according to the method of Monnet and Freney (11). Aqueous solutions of ethanolamine and histamine were prepared with 10 g of carbon

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TABLE 1. Biochemical reactions for 352 stock and 84 clinical isolates of seven *Klebsiella* species

Klebsiella isolates	No. strains tested	Test results (no. [%] positive reactions) for:						
		Ethanolamine	Histamine	D-Melezitose	DL-3 Hydroxybutyrate	Indole	Ornithine[a]	Sorbose
Stock								
K. pneumoniae	205	204 (99)	0	0	205 (100)	0	4 (2)	98 (48)
K. terrigena	61	3 (5)	58 (95)	60 (98)	61 (100)	0	3 (5)	42 (91)
K. planticola	26	0	25 (96)	3 (12)	23 (88)	7 (28)	0	4 (100)
K. ornithinolytica	20	0	20 (100)	2 (10)	20 (100)	20 (100)	20 (100)	20 (100)
K. oxytoca	20	20 (100)	0	20 (100)	0	20 (100)	0	20 (100)
K. ozaenae	10	2 (20)	0	0	3 (30)	0	0	7 (70)
K. rhinoscleromatis	10	7 (70)	0	0	7 (70)	0	0	0
Clinical								
K. oxytoca	7	7 (100)	0	7 (100)	1 (14)	NA[b]	NA	6 (86)
K. pneumoniae	77	76 (99)	0	0	77 (100)	NA	NA	42 (55)

[a] Ornithine results calculated from 196 strains. Ornithine was not routinely tested before 1978.
[b] Results not available.

substrate per liter. D-Melezitose and DL-3-hydroxybutyrate aqueous solutions were prepared by using 20 g of carbon substrate per liter. The solutions were filter sterilized through 0.22-μm-pore-size Millex filters (Millipore Corp., Bedford, Mass.) and were stored at 4°C in 50-μl aliquots in 13- by 100-mm sterile screw-cap tubes. A fifth tube without carbon substrate was used as a growth control. Two hundred microliters of AUX medium (bioMérieux, Inc.) was added to each tube prior to use. All tubes were inoculated with 100 μl of a 0.5 McFarland suspension of the respective strain made up in 0.85% saline. All tubes were incubated at 30°C and were examined at 24 and 48 h. Tubes with growth greater than or equal to the control tube were considered positive.

Utilization of *m*-hydroxybenzoate as the sole carbon source was determined by following the method of Naemura (14). The basal salt medium was prepared from three solutions. Solution A contained 10 g of Tris-HCl, 2 g of NH_4Cl, 2 g of KCl, 1 g of KH_2PO_4, 0.1 g of Na_2SO_4, and 900 ml of distilled water and was autoclaved prior to use. Solution B was prepared by mixing 100 ml of distilled water with 0.05 g of $CaCl_2 \cdot 2H_2O$ and 0.02 g of $MgCl_2$ and then autoclaving. Solution C contained 1 g of *m*-hydroxybenzoate dissolved in 100 ml of distilled water and was filter sterilized through a 0.22-μm-pore-size Millex filter. The final test medium consisted of 90 ml of solution A, 1 ml of solution B, and 10 ml of solution C. Aliquots of 200 μl were pipetted into sterile 13- by 100-mm screw-cap tubes and were stored at 5°C until used. A growth control without carbon source was prepared by mixing 90 ml of solution A and 1 ml of solution B; this mixture was then aliquoted into 200-μl samples. A set of two tubes (one with carbon substrate and one growth control) was inoculated for each organism with 100 μl of a 0.5 McFarland suspension of the organism in 0.85% NaCl. The basal salt medium with 0.1% *m*-hydroxybenzoate was incubated for 6 days at 35°C. Tubes with growth greater than that of the control tube were considered positive.

Pectate degradation. Liquefaction of pectate was determined by the method of Ewing (4).

Pigmentation on D-gluconate ferric citrate agar. Observation for brown pigment was determined according to methods used by Naemura (14).

Production of indole. Peptone water (2%) was inoculated, incubated at 35°C for 48 h, and developed with Kovac's reagent (4).

Fermentation of carbohydrates. Fermentation of D-melezitose, L-sorbose, and dextrose was determined by using Enteric Fermentation Base (BBL Microbiology Systems) with Andrade's indicator. A 0.5% filter-sterilized solution of either D-melezitose or L-sorbose was added to the base. Commercially obtained Andrade's broth with 1% dextrose (BBL) was also used. All tubes were inoculated with a light suspension of each respective organism in nutrient broth. All tests were incubated at 35°C and read daily for 7 days. For temperature-dependent tests at 5 ± 1°C, the tubes were incubated for 7 days in a refrigerator and read daily (4).

Ornithine decarboxylase. Commercially obtained Moeller's ornithine and paired base (BBL) was used. All tubes were inoculated with a light suspension of each respective organism in nutrient broth and were overlaid with approximately 1 in. of sterile mineral oil. They were incubated at 35°C and read daily for 7 days.

Fecal coliform reaction. EC broth (Difco) was inoculated with a light suspension of organism and was incubated for 24 h at 44.5 ± 0.5°C (1). Tubes producing gas were considered to be positive for fecal coliform.

RESULTS AND DISCUSSION

Expected results of carbon source, indole, and ornithine testing for seven species of *Klebsiella* are shown in Table 3. The results of carbon source utilization, indole, and ornithine testing with our strains of seven *Klebsiella* species essentially agreed with those of Monnet and Freney (11) (Table 1). *K. pneumoniae*, *K. oxytoca*, and *K. ornithinolytica* were easily and accurately identified by using these methods. An organism was identified as *K. planticola* if it was negative in tests for ethanolamine and ornithine decarboxylase and was positive in tests for histamine. An organism was identified as *K. terrigena* if it was negative in tests for indole and was positive in tests for D-melezitose. After testing 205 stock *K. pneumoniae* strains by using the additional substrates described in this paper, only one strain was identified as *K. planticola* and none was identified as *K. terrigena*. We found carbon source utilization testing to be less suitable for the identification of *K. ozaenae* and *K. rhinoscleromatis* because of the similarity in test results. During the testing of 10 strains of each species, there was poor growth, making the tests difficult to read and interpret. Fortunately, these two species are not often encountered in the United States. None of the fresh clinical isolates originally identified at local hospitals as *K. pneumoniae* or *K. oxytoca* were reidentified as *K. planticola* or *K. terrigena* by using carbon source assimilation testing.

For those laboratories wishing to identify *K. planticola* and

TABLE 2. Biochemical reactions (% positive) for *K. oxytoca* isolated from newborns

Tested species	Test results (no. [%] positive reactions) for:							
	Fecal coliform	Pectate	Pigment on ferric citrate	Melezitose fermentation	*meta*-Hydroxy-benzoate	Indole	Fermentation of glucose at 5°C	Sorbose
K. oxytoca (expected reactions[a])	0	100	74	74	98	100	0	98
K. planticola (expected reactions)	0	0	0	0	0	21	100	100
K. oxytoca (43 strains)	0	39 (91)	25 (58)	36 (84)	42 (98)	100	0	100

[a] Reactions from Monnet et al. (12) and Bagley et al. (2).

TABLE 3. Expected biochemical reactions for seven species of *Klebsiella*

Species	Reaction results (% positive) for:						
	Ethanolamine[a]	Histamine[a]	D-Melezitose[a]	DL-3 Hydroxybutyrate[a]	Indole[b]	Ornithine[b]	Sorbose[b]
K. pneumoniae	96	0	1	99	0	0	54
K. oxytoca	94	0	74	2	99	0	99
K. ozaenae	33	0	0	33	0	3	75
K. rhinoscleromatis	100	0	0	100	0	0	0
K. planticola	0	100	0	100	20	0	100
K. terrigena	0	100	100	100	0	20	100
K. ornithinolytica	0	100	0	100	100	100	100

[a] Data from Monnet and Freney et al. (11, 12).
[b] Data from CDC.

K. terrigena, the additional battery of tests proposed by Monnet and Freney is sensitive and specific.

Results of the biochemical tests on the 43 strains of *K. oxytoca* isolated from newborns (Table 2) confirmed the identities of 42 strains as *K. oxytoca*. We found only one strain that might possibly be identified as *K. planticola*, but it did not ferment glucose at 5°C, which it should have done. Only DNA hybridization would confirm its identity as *K. planticola*. An isolate was identified as *K. planticola* if it was negative in tests for pectate degradation, fermentation of melezitose, pigment production on ferric citrate agar, and utilization of *m*-hydroxybenzoate and failed to produce gas from lactose at 44.5°C. Our incidence does not agree with that of Podschun and Ullmann who reported that 9% of all *Klebsiella* spp. isolated from newborns in their institution were *K. planticola* (17). Because CDC is a reference laboratory that receives only problem isolates for identification, it is probable that we are not receiving a true surveillance set of isolates. Our entire collection of *K. oxytoca* (and, thus, of possible *K. planticola* isolates) numbers approximately 200 strains.

After retesting 479 strains, we could identify only two possible *K. planticola* strains. It appears that in the United States, *K. planticola* may not be present at the same rate as in other countries (0.5% versus 9 to 13%, respectively). At this time, the need for additional testing of *Klebsiella* isolates in clinical laboratories in the United States is not warranted.

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