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Recommended Test Panel for Differentiation of *Klebsiella* Species on the Basis of a Trilateral Interlaboratory Evaluation of 18 Biochemical Tests

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***Klebsiella pneumoniae* and *Klebsiella oxytoca* are the two most frequently encountered *Klebsiella* species giving rise to infections in humans, but other *Klebsiella* species can also be found in clinical specimens: *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis*, *Klebsiella terrigena*, *Klebsiella planticola*, *Klebsiella ornithinolytica*, and *Enterobacter aerogenes* (*Klebsiella mobilis*). However, many of these species are indistinguishable by the conventional methods employed routinely in the clinical microbiological laboratory. Several investigators have suggested various additional tests, but as yet there is no standardized test panel for identifying all *Klebsiella* species and subspecies. In the present study, performed in three national *Klebsiella* reference laboratories, we have evaluated a test panel consisting of 18 biochemical tests on 242 strains comprising all *Klebsiella* species and subspecies. The test panel was designed to identify organisms preliminarily identified as belonging to the genus *Klebsiella* on the basis of conventional methods or automated identification systems. With the described test panel it is possible to find one or more positive test results differentiating any *Klebsiella* species, except *Klebsiella rhinoscleromatis*, from its closest relative.**

Klebsiella is among the five gram-negative pathogens most commonly encountered in hospital-acquired infections (11), and *Klebsiella pneumoniae* is the most frequently occurring species, accounting for 75 to 86% of *Klebsiella* species reported (3, 10, 29). Much more rarely encountered are *Klebsiella ozaenae* and *Klebsiella rhinoscleromatis*, which have been retained as separate species because of their association with specific diseases (24). Taxonomically, these two species are regarded as subspecies of *K. pneumoniae* based on DNA-DNA hybridization data (20). *Klebsiella oxytoca* is the other well-established species, accounting for 13 to 25% of isolates (3, 10, 29).

The early 1980s saw the classification of environmental *Klebsiella* strains into provisional taxa (groups J, K, L, and M) (8) from which three new species have emerged: *Klebsiella terrigena* (12), *Klebsiella planticola* (1), and *Klebsiella ornithinolytica* (26). It has recently been proposed that these last three species be transferred to the new genus *Raoultella* (4), but as this classification has been controversial (2), we have chosen to use the older nomenclature in this report. The pathogenicity of these species for humans has been considered negligible, as they mainly have been isolated from environmental habitats. Recent studies, however, demonstrated that these new species can also be isolated from clinical specimens (14, 18, 21–23). *K. planticola*, in particular, has often been found, accounting for up to 18% of all clinical *Klebsiella* isolates in some studies (18, 21, 23), although this does not appear to be the case in the United States (30). Finally, the species *Enterobacter aerogenes* is often regarded as an eighth member of the genus *Klebsiella* (*Klebsiella mobilis*) (20).

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By the conventional methods used routinely in the microbiological laboratory, the new species are largely indistinguishable from *K. pneumoniae* and *K. oxytoca*. Several authors have suggested additional tests (1, 12, 16, 26), but as yet there is no standardized test panel for identifying all *Klebsiella* species and subspecies.

The present paper describes such a test panel and its evaluation by three national *Klebsiella* reference laboratories from different European countries (Denmark, Germany, and the United Kingdom). The test panel was designed to identify organisms preliminarily identified as belonging to the genus *Klebsiella* on the basis of conventional identification methods and consisted of 14 tests reported in the literature as discriminatory for *Klebsiella* spp. (1, 6, 12, 16, 17, 20, 26, 28), supplemented with the fermentation of four carbohydrates that showed promising results in preliminary experiments (9). For validation, the eight known *Klebsiella* species and subspecies were used, together with *Pantoea agglomerans*, as this species in our experience is often confused with the less reactive *Klebsiella* species. As three laboratories participated in this validation, an interlaboratory comparison of the variability of the test results was also possible.

MATERIALS AND METHODS

Laboratories. Participants in this study were the National Reference Laboratory for *Klebsiella* Species, Department of Medical Microbiology, University of Kiel, Germany (NRL); the Central Public Health Laboratory, Colindale, London, England (CPHL); and The International *Escherichia* and *Klebsiella* Reference Centre (World Health Organization), Statens Serum Institut, Copenhagen, Denmark (SSI). All three laboratories have in practice national and international reference functions and have cooperated since 1995 in a trilateral quality assurance program on K typing.

Strains. A total of 242 strains were included in the study: *K. pneumoniae* (n = 28), *K. ozaenae* (n = 27), *K. rhinoscleromatis* (n = 10), *K. oxytoca* (n = 30), *K. terrigena* (n = 29), *K. planticola* (n = 31), *K. ornithinolytica* (n = 27), *E.*

TABLE 1. Biochemical tests and methods used by the three participating laboratories[a]

Biochemical test	Protocol (exceptions)		
	CPHL	NRL	SSI
Lysine decarboxylase	Micro	Macro	Macro (6 days)
Ornithine decarboxylase	Micro	Macro	Macro (6 days)
Indole	Micro	MacroA	Macro (1–2 days)
Methyl red	Micro	MacroA (1 and 5 days)	Macro (1–2 days)
Voges-Proskauer	Micro	MacroA	Macro (1–2 days)
Growth at 10°C	Plate (2 days)	MacroA (5 days)	Macro (3 days)
Gas from lactose, 44.5°C	Macro	Macro	Macro (+ bile[b])
Malonate	Micro	Macro	Macro (14 days)
Fermentation of:			
D-Arabinose	Micro	Micro (1–2 days)	Macro (14 days)
β-Gentiobiose	Micro	Micro (1–2 days)	Micro (2 days)
D-Melizitose	Micro	Macro (1–2 days)	Macro (14 days)
2-Deoxy-D-ribose	Micro	Micro (1–2 days)	Micro (2 days)
L-Sorbose	Micro	Macro (1–2 days)	Macro (14 days)
D-Tagatose	Micro	Micro (1–2 days)	Micro (2 days)
Utilization of:			
m-Hydroxy-benzoate	Plate (2 days)	Plate (1 + 3 days, 20–25°C)	Macro (3 days, 30°C)
Histamine	Plate (2 days)	Plate (1 + 3 days, 20–25°C)	Macro (3 days, 30°C)
Hydroxy-L-proline	Plate (2 days)	Plate (1 + 3 days, 20–25°C)	Macro (3 days, 30°C)
Degradation of pectate	Plate (2 days)	Plate (1–2 days)	Plate (3 days)

[a] Test were incubated at 35 to 37°C overnight (18 to 24 h), with exceptions noted in parentheses. Micro, in-house test in microwells; Macro, test in 3 to 10 ml of liquid media; MacroA, test in 3 to 5 ml of solid media; Plate, test on agar plate.
[b] Incubated for 18 h.

aerogenes (n = 30), and *P. agglomerans* (n = 30). Strains were supplied by all three laboratories and were predominantly of clinical origin (blood, urine, sputum, and fecal). The *P. agglomerans* strains were from a previously published study (13). Much care was taken to include only validated strains of each species. This was done either by extensive biochemical characterization by the providing laboratory or by including strains from official culture collections or from published studies with well-characterized strains. The type strains for all nine species and subspecies examined were also included: ATCC 13883 (*K. pneumoniae*), NCTC 5050 (*K. ozaenae*), ATCC 13844 (*K. rhinoscleromatis*), ATCC 13182 (*K. oxytoca*), ATCC 33257 (*K. terrigena*), ATCC 33531 (*K. planticola*), JCM 6096 (*K. ornithinolytica*), NCTC 10006 (*E. aerogenes*), and NCTC 9381 (*P. agglomerans*).

Biochemical methods. The methods used by each of the three laboratories for the 18 biochemical tests used in this study are shown in Table 1. In order to provide the most realistic estimate of test variability, each laboratory deliberately continued to use its standard method for each test, since complete standardization of biochemical methods is uncommon and, indeed, many reports do not provide sufficient detail to do so. Incubation periods for the various tests are shown in Table 1.

The following eight conventional biochemical tests were done according to well-established methods as described by Edwards and Ewing (5): lysine (LDC) and ornithine decarboxylation (ODC), indole, methyl red (MR), Voges-Proskauer (VP), malonate, and fermentation of D-melizitose and L-sorbose. At CPHL these tests were done with the same media composition but with an in-house microtray format (100 μl).

Fermentation of four additional carbohydrates, D-arabinose (Sigma A6085 at CPHL and Merck 4592 at NRL), β-gentiobiose (Sigma G3000 at CPHL, SSI, and NRL), 2-deoxy-D-ribose (Sigma D2751 at CPHL, NRL, and SSI), and D-tagatose (Sigma T2751 at CPHL, NRL, and SSI), were adapted to established microwell in-house tests in each of the three laboratories, with the one exception that D-arabinose (Merck 1494) fermentation at SSI was performed in a (macro) test tube.

At SSI the basal medium consisted of 10.0 g of peptone, 5.0 g of meat extract, 3.0 g of NaCl, 2.0 g of $Na_2HPO_4 \cdot 12H_2O$, 0.024 g of bromthymol blue, and distilled water up to 1,000 ml. At CPHL the basal medium consisted of 4.0 g of peptone 140, 10.0 g of NaCl, 0.6 g of K_2HPO_4, 0.32 g of bromthymol blue, 0.32 g of thymol blue, and distilled water up to 1,800 ml. At NRL the basal medium consisted of 25.0 g of nutrient broth powder (No. 2, Oxoid CM0067), 0.024 g of bromthymol blue, and distilled water up to 1,000 ml. The percentages of carbohydrate used and reaction volumes were 0.75% and 15 μl, 1.0% and 100 μl, and 1.0% and 100 μl for SSI, CPHL, and NRL, respectively. The tests were incubated at 37°C and read after 1 (CPHL) or 2 days (NRL and SSI).

At SSI growth at 10°C was tested according to the method of Naemura & Seidler (19) by inoculating a nutrient broth with a 10-μl loop from an overnight lactose broth. The CPHL incubated a nutrient agar plate for 48 h and 5 days after inoculation from an overnight nutrient broth. NRL performed this test on nutrient agar slants. After the application of a large inoculum by an inoculation needle, the tubes were incubated up to 5 days.

The fecal-coliform test, measuring the production of gas from lactose at 44.5°C, was performed at SSI in a bile-lactose broth as described elsewhere, with an incubation time of 18 h (25). At NRL, a lactose broth (Merck 1.10689.0500) was incubated for 24 h, and at CPHL a lactose broth was incubated overnight.

Utilization of histamine, hydroxy-L-proline, and *m*-hydroxy-benzoate were examined at NRL and CPHL on agar plates containing 0.4% of the respective substrate as a sole carbon source and 50 mg of ferric citrate per 1,000 ml. The basal agar medium consisted of 4.0 g of KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 5.0 g of $(NH_4)_2SO_4$, and 20.0 g of agar per 1,000 ml, as described by Lütticken et al. (15). The following substrates were used: *m*-hydroxybenzoic acid (Sigma H6761,), *trans*-hydroxy-L-proline (CPHL, Sigma H6002; NRL, Merck 7434), and histamine (Sigma H7250). After overnight incubation at 37°C, plates were further incubated for 72 h at room temperature, as this enhances the response of the environmental species such as *K. terrigena* and *K. planticola*. At SSI, these tests were performed as a macrotube modification (quadruple volume) of the method of Monnet et al., which allowed reading of the test by a densitometer (16). Histamine (Sigma H7250), hydroxy-L-proline (Merck 104506), and *m*-hydroxybenzoate (Sigma H 6761) were used in carbon concentrations of 0.14, 0.2, and 0.1%, respectively. The tests were incubated at 30°C for 3 days.

In all three laboratories the degradation of pectate was performed as described by Starr et al., with the modification that 2.0 g of pectate and 1.0 g of agar were used instead of 3.0 g of pectate (27). The strains were spotted or streaked onto the pectate-agar plates and incubated at 37°C for 3 days.

RESULTS

The percentages of strains positive for each test and species combination are given in Tables 2 and 3 for each of the three laboratories. The numbers of tests in which all three centers found all strains of a species to be negative or positive varied from 4 for *K. ozaenae* and 5 for *K. pneumoniae* to 10 for *K. ornithinolytica*. Conversely, the number of tests for which the mean of each triplet of results was between 11 and 89% (i.e., a variable result) ranged from two for *K. planticola* to six for *K. pneumoniae*, suggesting that for this panel of tests, *K. pneumoniae* is the least tightly defined species.

To have a more simple and usable *Klebsiella* speciation table,

TABLE 2. Percentages of positive samples of five *Klebsiella* species by laboratory and test[a]

Biochemical test	*K. pneumoniae* (*n* = 28)			*K. ozaenae* (*n* = 27)			*K. rhinoscleromatis* (*n* = 10)			*K. oxytoca* (*n* = 30)			*K. terrigena* (*n* = 29)		
	CPHL	NRL	SSI	CPHL	NRL	SSI	CPHL	NRL	SSI	CPHL	NRL	SSI	CPHL	NRL	SSI
Lysine decarboxylase	89.3	85.7	96.4	25.9	29.6	25.9	0.0	0.0	10.0	100.0	96.7	100.0	100.0	100.0	100.0
Ornithine decarboxylase	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Indole	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	90.0	100.0	100.0	0.0	0.0	0.0
Methyl red	10.7	3.6	10.7	85.2[m]	92.6	63.0	90.0[m]	30.0	90.0	40.0[m]	0.0	63.3	27.6[m]	62.1	96.6
Voges-Proskauer	96.4	96.4	96.4	3.7	3.7	7.4	0.0	0.0	10.0	100.0	100.0	100.0	86.2	89.7	100.0
Growth at 10°C	57.1[m]	0.0	0.0	14.8[m]	0.0	0.0	0.0	0.0	0.0	93.3	93.3	86.7	100.0	100.0	100.0
Gas from lactose 44.5°C	89.3	89.3	89.3	11.1	11.1	3.7	0.0	0.0	10.0	0.0	6.7	3.3	0.0	0.0	0.0
Malonate	89.3	100.0	96.4	11.1	7.4	3.7	100.0	100.0	100.0	100.0	93.3	96.7	96.6	93.1	93.1
Fermentation of:															
D-Arabinose	0.0[m]	57.1	60.7	0.0[m]	22.2	29.6	0.0[m]	10.0	20.0	86.7[m]	96.7	100.0	0.0	0.0	3.4
β-Gentiobiose	100.0[m]	75.0	100.0	70.4[m]	66.7	100.0	10.0[m]	50.0	10.0	100.0	100.0	100.0	100.0	100.0	100.0
D-Melizitose	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	73.3	66.7	70.0	100.0	100.0	100.0
2-Deoxy-D-ribose	7.1	3.6	10.7	0.0[m]	11.1	7.4	0.0[m]	0.0	60.0	0.0[m]	0.0	16.7	96.6	96.6	96.6
L-Sorbose	64.3	57.1	75.0	44.4	55.6	44.4	0.0[m]	20.0	30.0	100.0	100.0	100.0	89.7	89.7	93.1
D-Tagatose	42.9	28.6	42.9	0.0[m]	25.9	0.0	10.0	20.0	20.0	90.0	86.7	90.0	17.2	13.8	13.8
Utilization of:															
m-Hydroxy-benzoate	0.0	0.0	0.0	0.0	0.0	7.4	0.0	0.0	10.0	100.0	96.7	100.0	41.4	65.5	82.8
Histamine	0.0	3.6	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.0	0.0	96.6	100.0	96.6
Hydroxy-L-proline	35.7	21.4	0.0	0.0	7.4	0.0[m]	0.0	20.0	6.7	0.0	0.0	10.3	41.4	37.9	
Degradation of pectate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	96.7	100.0	0.0	0.0	0.0

[a] Tests showing ≤80% agreement between laboratories are considered to be method (m) dependent, as indicated.

Table 4 was created by converting the percentage of positive results from one laboratory (SSI) into a set of symbols as used in the ASM Manual of Clinical Microbiology (7). Since the methods and test formats used in one of the two other laboratories (CPHL or NRL) may be more in agreement with methods implemented in other laboratories, reference values are provided in Tables 2 and 3.

The percentages of agreement between each pair of laboratories, by test and species, was calculated, and results showing ≤80% agreement are indicated in Tables 2 and 3. This occurred for only 25 of the 162 test and species combinations as follows: D-arabinose accounted for six, MR for five, β-gentiobiose and 2-deoxy-D-ribose for four combinations each; growth at 10°C, L-sorbose, D-tagatose, and hydroxy-L-proline accounted for one or two each. Thus, these are the tests with poor reproducibility, which may well be the most method dependent.

DISCUSSION

In the present study, we have evaluated a test panel comprising 18 biochemical tests on 242 strains comprising all *Klebsiella* species and subspecies in three national *Klebsiella* reference laboratories. Detailed results are provided in Tables 2

TABLE 3. Percentages of positive samples of four additional *Klebsiella* species by laboratory and test[a]

Biochemical test	*K. planticola* (*n* = 31)			*K. ornithinolytica* (*n* = 27)			*E. aerogenes* (*n* = 30)			*P. agglomerans* (*n* = 30)		
	CPHL	NRL	SSI	CPHL	NRL	SSI	CPHL	NRL	SSI	CPHL	NRL	SSI
Lysine decarboxylase	100.0	100.0	100.0	100.0	100.0	100.0	96.7	96.7	96.7	0.0	0.0	0.0
Ornithine decarboxylase	0.0	0.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0	3.3	0.0	0.0
Indole	71.0	74.2	74.2	100.0	100.0	100.0	0.0	0.0	0.0	0.0	10.0	0.0
Methyl red	93.5	100.0	100.0	100.0	100.0	100.0	0.0	0.0	10.0	36.7[m]	46.7	90.0
Voges-Proskauer	90.3	90.3	100.0	74.1	88.9	88.9	100.0	100.0	100.0	90.0	83.3	90.0
Growth at 10°C	100.0	100.0	96.8	100.0	92.6	96.3	40.0	53.3	33.3	83.3	100.0	93.3
Gas from lactose 44.5°C	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Malonate	100.0	100.0	100.0	100.0	100.0	100.0	100.0	93.3	96.7	100.0	100.0	96.7
Fermentation of:												
D-Arabinose	0.0[m]	0.0	35.5	3.7[m]	44.4	63.0	0.0	0.0	3.3	0.0	0.0	0.0
β-Gentiobiose	96.8	100.0	100.0	100.0	100.0	100.0	100.0	86.7	100.0	13.3[m]	6.7	70.0
D-Melizitose	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-Deoxy-D-ribose	100.0	100.0	100.0	100.0	96.3	100.0	0.0[m]	3.3	20.0	90.0	86.7	96.7
L-Sorbose	100.0	100.0	100.0	92.6	96.3	100.0	0.0	0.0	0.0	0.0	0.0	0.0
D-Tagatose	3.2	3.2	3.2	7.4[m]	25.9	14.8	20.0	16.7	10.0	0.0	6.7	0.0
Utilization of:												
m-Hydroxy-benzoate	0.0	0.0	0.0	0.0	0.0	0.0	83.3	80.0	86.7	0.0	0.0	0.0
Histamine	93.5	93.5	96.8	85.2	88.9	92.6	90.0	83.3	86.7	0.0	3.3	0.0
Hydroxy-L-proline	100.0	100.0	96.8	85.2	85.2	92.6	86.7	83.3	90.0	0.0	0.0	0.0
Degradation of pectate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] Tests showing ≤80% agreement between laboratories were considered to be method (m) dependent, as indicated.

TABLE 4. Differentiation of *Klebsiella* species according to the methods described for SSI[a]

Biochemical test	Result for species								
	K. pneumoniae	*K. ozaenae*	*K. rhinoscleromatis*	*K. oxytoca*	*K. terrigena*	*K. planticola*	*K. ornithinolytica*	*E. aerogenes*	*P. agglomerans*
Lysine decarboxylase	+	d	−	+	+	+	+	+	−
Ornithine decarboxylase	−	−	−	−	−	−	+	+	−
Indole	−	−	−	+	−	d	+	−	−
Methyl red	d	d	+	d	+	+	+	−	+
Voges-Proskauer	+	−	−	+	+	+	d	+	+
Growth at 10°C	−	−	−	d	+	+	+	d	+
Gas from lactose at 44.5°C	d	−	−	−	−	−	−	−	−
Malonate	+	−	+	+	+	+	+	+	+
Fermentation of:									
D-Arabinose	d	d	d	+	−	d	d	−	−
β-Gentiobiose	+	+	−	+	+	+	+	+	d
D-Melizitose	−	−	−	d	+	−	−	−	−
2-Deoxy-D-ribose	d	−	d	d	+	+	+	d	+
L-Sorbose	d	d	d	+	+	+	+	−	−
D-Tagatose	d	−	d	+	d	−	d	−	−
Utilization of:									
m-Hydroxy-benzoate	−	−	−	+	d	−	−	d	−
Histamine	−	−	−	−	+	+	+	d	−
Hydroxy-L-proline	d	−	d	−	d	+	+	+	−
Degradation of pectate	−	−	−	+	−	−	−	−	−

[a] Reference values for the other methods and test formats are given in Tables 2 and 3. −, 0 to 10% positive; d, 11 to 89% positive; +, 90 to 100% positive.

and 3 to allow the reader to choose between methods and test formats and still find relevant reference values.

Variable results were of two types: (i) those in which all three laboratories found a mixture of positive and negative results among the representatives of a species for a specific test (e.g., *K. pneumoniae* and L-sorbose), suggesting a truly variable reaction, and (ii) those in which one center found most strains to be negative while another found most positive (e.g., *K. rhinoscleromatis* and MR) or vice versa, suggesting that the results were method dependent. These method-dependent tests demonstrate that meticulous standardization is needed before results can be compared between laboratories and that without this standardization each laboratory must use method-adjusted reference values.

In the ASM Manual of Clinical Microbiology, only 6 of the 18 biochemical tests used in the present investigation are listed for *Klebsiella* species: indole, MR, VP, LDC, ODC, and malonate (7). The percentages of positive reactions for these six tests are within the range shown in Tables 2 and 3, except that in the present study ODC was negative for all strains of *K. ozaenae* and *K. terrigena* in all three laboratories, instead of being 3 and 20% positive, respectively.

Of the 18 biochemical tests used in this study, the following 13 tests are listed for *Klebsiella* species by Ørskov in Bergey's Manual, Tables 5.16 and 5.17 (20): LDC, ODC, indole, MR, VP, growth at 10°C, gas from lactose at 44.5°C, malonate, D-melizitose, L-sorbose, *m*-hydroxy-benzoate, hydroxy-L-proline, and pectate. The percentages of positive reactions for these 13 tests are within the ranges indicated in Tables 2 and 3, with the one exception that we find fewer (0 to 6.7%) *K. oxytoca*-positive isolates in hydroxy-L-proline than indicated in Table 5.16 (26 to 75%) of Bergey's Manual.

Several tests were identified that facilitated the differentiation of *P. agglomerans* strains from the less reactive *Klebsiella* species (*K. ozaenae* and *K. rhinoscleromatis*), namely, LDC, VP, malonate, growth at 10°C, and 2-deoxy-D-ribose fermentation.

In conclusion, based on our experience with this test panel, we suggest Table 4 as a scheme for identifying species within the *Klebsiella* genus. With the exception of the most biochemically inactive (sub-) species *K. rhinoscleromatis*, the suggested scheme makes it possible to find one or more positive test results differentiating any species from its closest relative. If the number of tests has to be reduced, MR, D-arabinose, D-tagatose, and D-sorbose could be omitted, as they are either method dependent or less discriminating than the remaining tests.

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