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Efficacy of the Ryu Nonstaining KOH Technique for Rapidly Determining Gram Reactions of Food-Borne and Waterborne Bacteria and Yeasts

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A simple and rapid (<60 s) nonstaining technique with 3% potassium hydroxide to determine Gram reactions was tested with 495 food-borne and waterborne bacteria and yeasts. In KOH, suspensions of gram-negative bacteria become viscous and string out. Gram-positive bacteria are not affected. There was 100% correlation between the KOH string test results and gram-positive and gram-negative strains.

A simple, nonstaining method, requiring only one reagent to determine the Gram reaction, was first reported in 1938 by E. Ryu of Taiwan (see references 13 and 14 [also cited in references 6, 7, 11, and 19]). In spite of some more recent evaluations (2–4, 6–12, 19), the method still appears to be obscure and not widely known among food and water microbiologists. The potassium hydroxide string test has been tested against veterinary isolates (6, 7), bacterium and yeast isolates from brewery samples (11), marine isolates (3, 18), *Listeria* spp., (10), clinical anaerobes (2, 4, 8), and other clinical isolates (9). The purpose of this study was to verify the efficacy of the KOH string test for determining the Gram reactions of food-borne and waterborne bacteria.

Test microorganisms included isolates from food, milk, and water and from stock cultures of microorganisms found in foods and water. Cultures were obtained from U.S. Army stock cultures, other government agencies (U.S. Department of Agriculture, Food and Drug Administration, and Centers for Disease Control and Prevention) food industries, hospitals, universities, and the American Type Culture Collection (Rockville, Md.) or were obtained directly from military foods analyzed.

The KOH string test was performed by mixing a visible amount of growth from a colony or an agar slant in a loopful (3 mm) of 3% aqueous KOH on a glass slide. The KOH-bacterium suspension was mixed continuously with a bacteriological loop in a 1- to 2-cm^2 area on the slide. If such a suspension gels or becomes viscous and strings out when the loop is lifted (positive KOH reaction), the isolate is gram negative. Gram-positive cells do not form a viscous gel or string out (negative KOH reaction). The cutoff time for negative reactions was 60 s. The string test was the best indication of a gram-negative isolate, and the result was best observed by raising the loop about 1 cm above the slide. Holding the slide at an angle against a dark background also aided the observation. Colonies from culture plates of food samples and from selective and differential media were transferred to Trypticase soy agar (Difco, Detroit, Mich.) or plate count agar (Difco) for both the KOH string test and Gram staining.

Gram staining (5, 15, 17) was performed on 18- to 24-h cultures in parallel with the KOH string test. Identification of gram-negative isolates was confirmed by using, singly or in combination, the API 20E system (Analytab Products, Plainview, N.Y.), the Enterotube system (Roche Diagnostic Systems Inc., Montclair, N.J.), and the Micro-ID system (General Diagnostics, Morris Plains, N.J.). Gram-positive isolates were examined microscopically for morphology and the presence of spores.

Table 1 compares the results of Gram staining with those of the KOH string test for 231 isolates from food, milk, dietary capsules, and water. There was no false reaction with the KOH string test, but two isolates (<1%) gave anomalous results with the Gram stain. One of these two isolates was *Clostridium thermosaccharolyticum*. This organism was identified by conventional microbiological methods and compared with a known *C. thermosaccharolyticum* reference strain, ATCC 7956. Both cultures of this organism always stained as gram negative, which is typical of this strain (16). However, the KOH string test was negative, which correctly identified *C. thermosaccharolyticum* as a gram-positive organism. The second equivocal isolate was *Enterobacter cloacae*, which stained as gram-positive but correctly gave the positive KOH string reaction of a gram-negative organism.

The KOH string test can be performed directly with colonies from Baird Parker agar (Difco), violet-red bile agar (Difco), and KF *Streptococcus* agar (Difco), but not with colonies from eosin-methylene blue agar (Difco). Some atypical colonies from eosin-methylene blue agar gave false KOH reactions and Gram stains. Colonies of *Klebsiella oxytoca* 34763 (Enterotube ID value) tested directly from eosin-methylene blue agar were negative for the KOH test but were KOH positive as expected when tested from Trypticase soy agar. Colonies of gram-positive, rod-shaped bacteria on eosin-methylene blue agar were mucoid and stringy to begin with (false-positive reaction), but the same isolates on Trypticase soy agar and plate count agar were not and reacted correctly.

The KOH reactions of 24-h bacterial stock cultures are presented in Table 2. There were no false-positive or false-negative KOH reactions among the 91 gram-positive and 173 gram-negative strains (57 species). Fourteen-day-old cultures of 70 gram-positive and 21 gram-negative bacteria also tested correctly with the KOH string test.

Table 3 presents the results of 11 other studies which evaluated the KOH string test with a total of 3,088 strains of bacteria and yeasts relevant to different disciplines. Most of the false KOH reactions were reported with clinical anaerobes (2, 4, 8) which were largely *Bacteroides* species. Carlone et al. (4) reported only one false-positive KOH reaction with a strain of *Bacillus cereus*. Only one veterinary isolate (*Bacillus macerans*) gave a false-positive reaction (7), and only one veterinary iso-

TABLE 1. Comparison of results of Gram staining and KOH reaction of food-borne and waterborne isolates[a]

Isolate(s)	No. of strains	No. of species	Gram stain	KOH reaction
Aeromonas hydrophila	1	1	Neg	Pos
Bacillus spp.	4	3	Pos	Neg
C. thermosaccharolyticum	1	1	**Neg**	**Neg**
Citrobacter sp.	1	1	Neg	Pos
Enterobacter spp.	9	3	Neg	Pos
E. cloacae	1	1	**Pos**	**Pos**
Escherichia coli	21	1	Neg	Pos
Flavobacterium sp.	1	1	Neg	Pos
K. oxytoca	5	1	Neg	Pos
Non-spore-forming rods	42[b]	NI[c]	Neg	Pos
Non-spore-forming rods	11	NI	Pos	Neg
Lactobacillus spp.	6	3	Pos	Neg
Pseudomonas spp.	6	3	Neg	Pos
Sarcina lutea	4	1	Pos	Neg
Serratia liquefaciens	1	1	Neg	Pos
Spore-forming bacilli	94	NI	Pos	Neg
Staphylococcus spp.	13	2	Pos	Neg
Streptococcus faecalis	2	NI	Pos	Neg
Saccharomyces sp. (yeast)	8	NI	Pos	Neg
Total	231	>23		
% correct reactions			99.1	100

[a] Boldface indicates identical results obtained by Gram stain and the KOH reaction. Neg, negative; Pos, positive.
[b] Includes 11 waterborne isolates.
[c] NI, not identified.

late gave a false-negative reaction (6). There were no false KOH reactions reported with brewery cultures (11), *L. monocytogenes* and other *Listeria* spp. (10), marine cultures (3), and clinical isolates tested by Kohn and Henneman (9). A cutoff time of only 10 s was used for the KOH reaction by Gregersen (7) and von Graevenitz and Bucher (19), and a cutoff time of only 30 s was used by several other investigators (2, 4, 8, 12). False-negative KOH reactions were reduced by increasing the cutoff time for negative reactions to 60 s (6, 19). In this study, some of the gram-negative food-borne bacteria were negative for the KOH string test after 30 s but were positive as expected after 35 to 40 s and longer. Therefore, the cutoff time for negative KOH string tests should not be less than 60 s. The low percentage of false reactions with 3,088 diverse strains of bacteria (Table 3) indicates that the KOH string test is a very useful and reliable supplement to, if not a substitute for, the Gram stain.

Unlike Gram staining, the KOH string test can be performed on cultures more than 24 h old (6, 7). Gram-negative bacteria produce a positive KOH reaction because their cell walls are easily disrupted when exposed to dilute alkali solutions (2, 7, 8, 12). When the cell walls are disrupted in 3% KOH, the suspension becomes viscous within 5 to 60 s and strings out because of the release of unfragmented threads of DNA (2, 4, 7, 8, 12). The cell walls of gram-positive bacteria are not affected by 3% KOH (7, 8).

False-negative KOH reactions with gram-negative bacteria can result from an incorrect Gram stain (1, 2, 4, 7, 19), insufficient reaction time (<60 s), insufficient inoculum (11), dilution by too much KOH solution, and improper growth medium. False-positive KOH reactions with gram-positive bacteria can occur with too much inoculum (the suspension appears to gel when mixed with KOH but does not string), incorrect Gram stain, mixed culture (6, 11), improper growth medium, and inocula from mucoid, sticky colonies. Inocula from the sticky, mucoid centers of colonies produced by some gram-positive spore-forming bacteria formed a string even in controls with distilled water. However, when dry material from the edge of the colony was mixed in a 3% KOH solution, the KOH string reaction was negative as expected.

TABLE 2. KOH reactions of bacterium and yeast stock cultures[a]

Microorganism	No. of strains	No. of species	Gram stain	KOH reaction
Bacillus cereus	3	1	Pos	Neg
Bacillus spp.	7	3	Pos	Neg
Clostridium perfringens	4	1	Pos	Neg
Clostridium sporogenes	2	1	Pos	Neg
C. thermosaccharolyticum	2	1	Neg	Neg
Gaffkya tetragena	1	1	Pos	Neg
Listeria spp.	59	3	Pos	Neg
Micrococcus lysodeikticus	1	1	Pos	Neg
Saccharomyces spp. (yeast)	5	2	Pos	Neg
Staphylococcus aureus	6	1	Pos	Neg
Streptococcus faecalis	1	1	Pos	Neg
A. hydrophila	2	1	Neg	Pos
Citrobacter spp.	4	3	Neg	Pos
Campylobacter jejuni ATCC 33292	1	1	Neg	Pos
Enterobacter spp.	16	4	Neg	Pos
E. coli	77	1	Neg	Pos
Klebsiella spp.	30	4	Neg	Pos
Moraxella catarrhalis	1	1	Neg	Pos
Proteus spp.	5	4	Neg	Pos
Providencia alcalifaciens	1	1	Neg	Pos
Pseudomonas aeruginosa	1	1	Neg	Pos
Salmonella spp.	25	15	Neg	Pos
Serratia marcescens	4	1	Neg	Pos
Shigella dysenteriae	1	1	Neg	Pos
Spirillum serpens	1	1	Neg	Pos
Vibrio parahaemolyticus	2	1	Neg	Pos
Yersinia enterocolitica	2	1	Neg	Pos
Total	264	57		
% Correct reactions			99.2	100

[a] Neg, negative; Pos, positive.

TABLE 3. Differentiation of gram-positive and gram-negative microorganisms by KOH reaction

Reference	Isolates or nature of isolates	No. of strains	% False-positive KOH reactions	% False-negative KOH reactions
2	Clinical anaerobes	931	3	10
8	Clinical anaerobes	213	0	28
4	Aerobes, anaerobes	128	11[a]	8
12	Clinical, water, milk	146	2	2
7	Veterinary	126	1	0
19	Medical, miscellaneous	488	8	9
9	Clinical	100	0	0
6	Veterinary	69	0	2
11	Brewery	444	0	0
3	Marine	370[b]	0	0
10	*Listeria*, nonlisterial	73	0	0
Total		3,088		
Avg %			2	5

[a] The 11% false-positive result is due to one false-positive reaction out of nine.
[b] Excludes 30 strains consisting of *Corynebacterium* and *Arthrobacter* species which are typically Gram variable (3). These cultures reportedly gave some equivocal KOH results, but percent false reactions could not be determined from the report (3).

The KOH string test was very accurate and reliable for 495 food-borne and waterborne microorganisms. The Ryu KOH string test is endorsed and presented to food microbiologists and others as a useful, rapid, and simplified method to determine Gram reactions without staining.

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