

# Evaluation of the KOH Test and the Antibiotic Disk Test in Routine Clinical Anaerobic Bacteriology

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Received 28 April 1988/Accepted 11 July 1988

We have evaluated the KOH test, the antibiotic disk identification test, and the Gram stain reaction for the preliminary grouping of gram-positive and gram-negative anaerobes and have assessed the value of erythromycin 60- $\mu$ g-disk resistance as a predictive index of clindamycin resistance among anaerobes. By testing 931 clinical isolates, 281 gram positive and 650 gram negative, with the KOH test and vancomycin 5- $\mu$ g-disk test, we obtained the following parameters: sensitivity, 89.7 and 97.1%; specificity, 97.5 and 98.3%; positive predictive value, 80.4 and 98.7%; and efficiency, 92.1 and 98% for the KOH test and the vancomycin test, respectively. The KOH reaction incorrectly grouped 42 of 97 *Bacteroides bivius* and 12 of 50 pigmented *Bacteroides* strains. The vancomycin test correctly identified 63 of 67 gram-negative strains that had given a negative KOH reaction. The erythromycin disk result correctly predicted clindamycin resistance in gram-negative isolates but had a sensitivity of 85.7%, a specificity of 92.4%, and a positive predictive value of 42.8% for gram-positive isolates. Therefore, the use of these preliminary identification tests can assist in the correct grouping of anaerobes and accurately predict significant clindamycin resistance in gram-negative anaerobic bacteria.

The initial classification of an unknown bacterium and subsequent identification procedures are largely based on the results of the Gram stain. The major pitfall in the Gram staining technique is the tendency of some gram-positive bacteria, particularly anaerobes, to decolorize more readily than others, which often results in these bacteria being classified incorrectly as gram negative. Two other rapid techniques can aid in distinguishing between gram-positive and gram-negative organisms, the antibiotic susceptibility pattern (6) and the less commonly known KOH solubility reaction (2). As an additional advantage of the antibiotic susceptibility test, Murray and Weber (Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 166, 1983) have suggested that resistance to the erythromycin 60- $\mu$ g disk used in the antibiotic panel could accurately predict clindamycin resistance among isolates of the *Bacteroides fragilis* group.

We report herein our experience with the use of the two tests described above in the routine identification of anaerobic bacteria. Our specific objectives were to evaluate the relative value of each test in the correct classification of gram-positive and gram-negative anaerobic bacteria and to assess the value of erythromycin resistance as a predictive index of clindamycin resistance.

## MATERIALS AND METHODS

**Bacterial strains.** A total of 931 anaerobic clinical isolates, including 281 gram-positive and 650 gram-negative organisms, was examined. The bacteria were identified by the methods outlined in the *Wadsworth Anaerobic Laboratory Manual* (5). Gas-liquid chromatography was performed by the methods in the Virginia Polytechnic Institute manual (3).

**Antibiotic disk susceptibility tests.** For the antimicrobial disk tests, disks of vancomycin (5  $\mu$ g), colistin (10  $\mu$ g), and erythromycin (60  $\mu$ g) (An-Ident Discs; Oxoid Ltd., Basingstoke, Hampshire, England) were placed onto the surface of

an inoculated blood agar plate. The plates were incubated at 37°C for 24 to 48 h in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). Susceptibility to the antimicrobial agent was defined as any zone of inhibition after 24 to 48 h of incubation (5).

**KOH solubility reaction.** The KOH test was performed by the method of Halebian et al. (2). Briefly, 2 drops of a 3% solution of potassium hydroxide was placed on a glass slide. A 2-mm loopful of bacterial growth was stirred in a circular motion in the KOH solution. The KOH solution characteristically became very viscous and mucoid with gram-negative bacteria. A string of the mixture would follow the loop when it was raised. The KOH reaction was considered positive if stringing occurred within the first 30 s of mixing the bacteria in the solution.

**Clindamycin susceptibility testing.** The MICs of clindamycin were determined for 333 isolates by the National Committee for Clinical Laboratory Standards reference agar dilution procedure for susceptibility testing of anaerobic bacteria (4) with Wilkins-Chalgren agar (7). The clindamycin resistance breakpoint was defined as an MIC of  $\geq 8$   $\mu$ g/ml.

**Evaluation of the tests.** The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficacy of each test were calculated (1).

## RESULTS

Table 1 summarizes the results of the KOH solubility reaction and the vancomycin and colistin disk patterns for the 281 gram-positive isolates examined. The profile obtained was highly consistent: negative KOH reaction (97.5%), susceptibility to vancomycin (97.2%), resistance to colistin (98.5%), and combined susceptibility to vancomycin and resistance to colistin (95.7%). Only seven strains gave false-negative KOH reactions: five *Clostridium clostridioforme*, one *Clostridium cadaveris*, and one *Propionibacterium acnes*.

The results obtained with the gram-negative isolates are

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TABLE 1. Results of the KOH reaction and of the antibiotic disk susceptibility test for 281 gram-positive bacteria

Genus	Tested	No. of strains			
		KOH negative	Vancomycin susceptible <sup>a</sup>	Colistin resistant <sup>b</sup>	Vancomycin susceptible and colistin resistant
<i>Clostridium</i>	198	192	190	197	189
<i>Peptostreptococcus</i>	39	39	39	37	37
<i>Propionibacterium</i>	22	21	22	21	21
<i>Lactobacillus</i>	8	8	8	8	8
<i>Bifidobacterium</i>	6	6	6	6	6
<i>Actinomyces</i>	3	3	3	3	3
<i>Eubacterium</i>	5	5	5	5	5
% of total		97.5	97.2	98.5	95.7

<sup>a</sup> 5- $\mu$ g disk.<sup>b</sup> 10- $\mu$ g disk.

shown in Table 2. Only 583 (89.7%) of the 650 gram-negative bacteria tested gave a positive KOH reaction, but 98.3% were resistant to vancomycin. As expected, the colistin susceptibility pattern was more variable with the gram-negative organisms than with the gram-positive organisms. Sixty-seven strains (10.3%) gave a false-negative KOH reaction. These were *Bacteroides bivius* (42 strains), pigmented *Bacteroides* spp. (12 strains), *Bacteroides* spp. (8 strains), *Veillonella* spp. (3 strains), and the *B. fragilis* group (2 strains). The KOH test therefore had an overall sensitivity of 89.7%, specificity of 97.5%, PPV of 98.2%, NPV of 80.4%, and efficacy of 92.1%. Similarly, considering that 273 of the 281 gram-positive isolates were susceptible to vancomycin (true-positive) and that 639 of the 650 gram-negative isolates were resistant to vancomycin (true-negative), the vancomycin disk test had a sensitivity of 97.1%, specificity of 98.3%, PPV of 96.1%, NPV of 98.7%, and efficacy of 98%.

To assess further the value of the vancomycin susceptibility results in the preliminary identification of anaerobes, we have analyzed the results of the vancomycin disk test for the 331 KOH-negative isolates, that is, the 274 truly negative gram-positive isolates and the 67 falsely negative gram-negative isolates. Under these circumstances, the vancomycin susceptibility profile correctly classified 273 gram-positive strains (true-positive) and 63 of 67 gram-negative strains (true-negative). In this particular instance, the vancomycin disk test had a sensitivity of 98.5%, specificity of 97.4%,

TABLE 2. Results of the KOH reaction and of the antibiotic disk susceptibility test for 650 gram-negative bacteria

Organism(s)	Tested	No. of strains		
		KOH positive	Vancomycin resistant	Colistin response <sup>a</sup>
<i>B. fragilis</i> group	422	420	421	417 R
<i>B. bivius</i>	97	55	94	87 R
Pigmented <i>Bacteroides</i> spp.	50	38	46	41 R
Other <i>Bacteroides</i> spp.	32	24	30	18 R
<i>Fusobacterium</i> spp.	29	29	29	28 S
<i>Veillonella</i> spp.	20	17	19	19 S
% of total		89.7	98.3	

<sup>a</sup> R, Resistant; S, susceptible.TABLE 3. Organism susceptibility according to results of the erythromycin disk susceptibility test and the clindamycin MICs for 112 gram-positive isolates<sup>a</sup>

Organism(s) (no. of strains tested)	No. of strains			
	Erythromycin		Clindamycin	
	Susceptible	Resistant	Susceptible	Resistant
<i>Clostridium</i> spp. (72)	65	7	67	5
<i>Peptostreptococcus</i> spp. (33)	27	6	31	2
<i>Propionibacterium acnes</i> (4)	4	0	4	0
<i>Eubacterium</i> spp. (2)	1	1	2	0
<i>Lactobacillus</i> spp. (1)	1	0	1	0

<sup>a</sup> Complete agreement for 104 strains.

PPV of 98.1%, NPV of 94%, and efficacy of 97.3% in correctly classifying the bacteria.

Both the erythromycin disk susceptibility results and the MICs of clindamycin were available for 331 isolates. Of the 112 gram-positive isolates tested (Table 3), 7 were resistant to clindamycin. Three strains of *C. clostridiiforme* were resistant to erythromycin but susceptible to clindamycin, and one strain of *Clostridium sporogenes* was susceptible to erythromycin but resistant to clindamycin. Among the *Peptostreptococcus* species, only four of the six erythromycin-resistant isolates were resistant to clindamycin. Overall, eight strains (7.1%) gave discordant results: seven falsely resistant (erythromycin resistant but clindamycin susceptible) and one falsely susceptible (erythromycin susceptible but clindamycin resistant). For the gram-positive isolates, the use of erythromycin disk resistance as a predictor of clindamycin resistance yielded the following results: sensitivity of 85.7%, specificity of 92.4%, PPV of 42.8%, NPV of 99%, and efficacy of 91.9%.

Among the 221 gram-negative organisms tested (Table 4), 11 isolates of the *B. fragilis* group were resistant to erythromycin and all were highly resistant to clindamycin. There was perfect agreement between the erythromycin and the clindamycin susceptibility results.

## DISCUSSION

Like the Gram stain reaction, the KOH test is based on differences in the chemistry of the bacterial cell wall. The cell walls of gram-negative bacteria are easily disrupted when exposed to dilute alkali solutions. When the cell walls are disrupted, the suspension in KOH becomes viscous because of the release of relatively unfragmented threads of DNA (2). All the gram-negative bacteria examined in this

TABLE 4. Organism susceptibility according to results of the erythromycin disk susceptibility test and the clindamycin MICs for 221 gram-negative isolates

Organism(s) (no. of strains tested)	No. of strains			
	Erythromycin		Clindamycin	
	Susceptible	Resistant	Susceptible	Resistant
<i>B. fragilis</i> group (181)	170	11	170	11 <sup>a</sup>
<i>Bacteroides</i> spp. (26)	26	0	26	0
<i>Fusobacterium</i> spp. (7)	7	0	7	0
<i>Veillonella</i> spp. (7)	7	0	7	0

<sup>a</sup> Perfect agreement.

investigation were correctly identified by the Gram stain. However, 10% of the gram-negative bacteria gave negative KOH reactions typical of gram-positive bacteria. In contrast, 11% of the gram-positive bacteria stained negatively with the Gram reaction; however, 97.5% gave appropriate KOH negative reactions. Halebian et al. (2) examined 89 gram-positive and 109 gram-negative anaerobic isolates by the KOH test. They obtained no false-positive KOH reactions and 28.4% false-negative KOH reactions. In their study, the KOH test had a sensitivity of 70.6%, specificity of 100%, PPV of 100%, NPV of 68.9%, and efficacy of 84.6%. The discrepancies between their results and ours can be explained by the different proportion of gram-positive and gram-negative isolates and the representation of different species within each of the two classes. Furthermore, it was interesting to note that 43.3% of the *B. bivius* and 24% of the pigmented *Bacteroides* isolates gave false-negative KOH reactions. Halebian et al. (2) had noted that all of their 18 strains of *Bacteroides melaninogenicus* subsp. *intermedium* and *B. melaninogenicus* subsp. *melaninogenicus* gave a false-negative KOH reaction, whereas 5 of the 6 *Bacteroides asaccharolyticus* strains gave positive KOH reactions. As all of our strains of pigmented *Bacteroides* spp. have not been identified to species, we are unable to compare our results with those of Halebian et al. (2).

The vancomycin disk susceptibility test was very helpful in correctly classifying the anaerobes, especially those giving negative KOH reactions. In this respect, our results were similar to those of Halebian et al. (2), who found 97.8% of their gram-positive isolates susceptible to vancomycin and 97.1% of their gram-negative isolates resistant to vancomycin.

The disks used in preliminary grouping of anaerobes serve as a Gram stain check but do not imply susceptibility of an organism for antibiotic therapy (6). However, more recently, Murray and Weber (23rd ICAAC) have proposed utilization of the erythromycin disk as a rapid screening test for detecting clindamycin resistance and have shown that 8 of 98 *Bacteroides* spp. isolates resistant to clindamycin showed no zone of inhibition around the erythromycin 60- $\mu$ g susceptibility disk. We observed similar results for the gram-negative

isolates. However, for the gram-positive isolates, susceptibility to the erythromycin disk was not a good predictor of clindamycin resistance, since a significant number of isolates resistant to the erythromycin disk were found susceptible to clindamycin by the reference agar dilution method.

In summary, the use of the KOH reaction is effective in the preliminary classification of anaerobes in the clinical laboratory and is a useful complement to the Gram stain and the antibiotic disk test. Whereas positive KOH reactions are highly specific for gram-negative organisms, the relatively high number of false-negative reactions among this group justifies the routine use of the vancomycin disk test for the strains with negative KOH reactions. The erythromycin disk result can accurately predict clindamycin susceptibility in both gram-positive and gram-negative bacteria and accurately predict clindamycin resistance among isolates of the *B. fragilis* group. However, confirmation of resistance to clindamycin is mandatory for gram-positive isolates.

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