JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1982, p. 547–553
0095-1137/82/040547-07$02.00/0

Vol. 15, No. 4

Differentiation of Coagulase-Positive and Coagulase-Negative Staphylococci by Lectins and Plant Agglutinins

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The screening of staphylococci with a panel of 14 lectins and extracts demonstrating lectin-like activity led to the development of a rapid agglutination slide test for the differentiation of certain coagulase-negative staphylococci and human strains of *Staphylococcus aureus*. The coagulase-negative staphylococci were agglutinated by agglutinins from *Mangifera indica*, *Triticum vulgaris*, and crude *Limulus polyphemus*. The test is rapid, requiring only 5 to 15 min to identify an unknown strain of staphylococci, as opposed to the 4 to 16 h required to perform the conventional tube coagulase test.

For species identification of staphylococci, the ICSB Subcommittee on Taxonomy of Staphylococci and Micrococci recommends the following tests: coagulase production, aerobic production of acid from sucrose, trehalose, and mannitol, phosphatase production, and sensitivity to novobiocin (38). These multiple criteria are used primarily by research and reference laboratories. In many laboratories, coagulase production is the only test used to identify *Staphylococcus aureus*. Organisms which do not produce coagulase are collectively referred to as coagulase-negative staphylococci. These isolates are not identified further by the majority of clinical laboratories. Coagulase production is detected by the tube test for free coagulase or the clumping factor slide test (9). There have been reports of difficulty in interpreting the tube coagulase test (30, 36), and false-negative (36, 40) and false-positive (2, 30, 36, 40) tests have been reported.

Lectins are proteins or glycoproteins of nonimmune origin with sugar specificity (13, 22, 35). Some plant extracts also contain substances which may agglutinate several types of erythrocytes (25). By strict definition, these "agglutinins" are not lectins because they do not possess carbohydrate specificities and are not proteins; nevertheless, the extracts are selective agglutinating agents (25). Lectin cell binding can elicit a variety of phenomena, including agglutination, mitogenesis, and cytotoxicity (35). Lectins also form precipitates with carbohydrate-containing macromolecules and have been used for their isolation and purification (7). Within the last 10 years, some researchers have demonstrated the ability of lectins to agglutinate certain species of bacteria (12, 18, 19, 21, 24, 26, 28, 39). The specificity of agglutination of bacteria by lectins resides in the unique cell surface structures of the bacteria interacting with the carbohydrate-specific lectins (1, 18, 19, 26, 29, 31, 33). Only recently has lectin agglutination of bacteria been used as a method for the definitive identification of clinical isolates. Wheat germ agglutinin (WGA; *Triticum vulgaris*) has been shown to specifically agglutinate *Neisseria gonorrhoeae* and not to agglutinate encapsulated *Neisseria meningitidis* (11, 33, 34).

The objective of these studies was to develop a rapid procedure for differentiating coagulase-negative and coagulase-positive staphylococci by use of lectins or agglutinins. Reference strains of staphylococci and fresh clinical isolates were screened for agglutination with a battery of agglutinins and lectins. Five agglutinins were reactive with some staphylococci, and these were further investigated for the selective agglutination of coagulase-negative and coagulase-positive staphylococci. An agglutination slide test which used *Mangifera indica* (mango) extract, WGA, and crude *Limulus polyphemus* (horseshoe crab) lectin was found to agglutinate 96% (79 of 82) of the coagulase-negative strains of staphylococci and none of the *S. aureus* strains with the exception of the surface-defective mutant *S. aureus* Wood 46.

MATERIALS AND METHODS

Reagents. All chemicals, salts, and sugars were of the highest grade available. *L. polyphemus* (crude), *T. vulgaris* (pure), and other routinely used lectins were purchased from E-Y Laboratory (San Mateo, Calif.). *M. indica* extract was prepared from the dried seeds of commercially purchased mangoes. The seeds were pulverized in a micromill (Chemical Rubber Co., Columbus, Ohio) and extracted for 2 h at room temperature in 10 times their weight of phosphate-buffered saline (PBS: 0.05 M potassium phosphate, 0.15 M

sodium chloride, pH 7.2). Insoluble material was removed by centrifugation, and the supernatant was dialyzed against 5 liters of distilled water. The crude *M. indica* extract was centrifuged and lyophilized. This extract agglutinated various types of erythrocytes (unpublished data). Characterization and biological properties of the agglutinin activity will be reported elsewhere (S. K. Davidson, R. J. Doyle, and K. F. Keller, manuscript in preparation). *Lens culinaris* (commercial lentil), *Diospyros* sp. (commercial persimmon), and *Bandeiraea simplicifolia* (Calbiochem, La Jolla, Calif.) agglutinins were prepared from defatted seed meal (23) by fractional precipitation with ammonium sulfate (final concentration of 60% saturation). The ammonium sulfate precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed as above against distilled water or PBS. The extracts were lyophilized and tested for hemagglutinating activity.

Microorganisms. The reference strains used were *S. aureus* ATCC 25923 and 25904, *S. epidermidis* ATCC 14990, and *S. saprophyticus* ATCC 13518. Other microorganisms tested included 39 clinically isolated strains of *S. aureus*, 41 strains of *S. epidermidis*, 9 strains of *Staphylococcus* spp., and *Micrococcus* spp. obtained from Jewish Hospital Microbiology Laboratory, Louisville, Ky. All 91 strains were maintained on Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, Md.). The clinically isolated microorganisms were identified by Gram stain, catalase production, glucose fermentation (8), oxidation and fermentation of mannitol (2%, wt/vol) in tubes of cysteine-Trypticase agar medium (BBL), coagulase production (37), acid production from 1% (wt/vol) maltose, mannose, sucrose, and trehalose in purple broth base (Difco Laboratories, Detroit, Mich.) (14), and phosphatase production (17).

Agglutination slide test for differentiation of coagulase-positive and coagulase-negative staphylococci. The agglutination slide test was performed by a modification of the procedures of Schaefer et al. (33). The staphylococci were removed from sheep blood agar with a cotton swab and suspended in PBS. The suspension was adjusted to approximate a no. 4 McFarland barium sulfate standard. *M. indica* extract and WGA were diluted in PBS, mixed, and clarified by centrifugation. The final concentration (dry weight) of each agglutinin was 125 μg/ml. One drop of the bacterial suspension was placed into each of two wells in a Boerner slide; one drop of the *M. indica* extract-WGA solution was added to well 1, and one drop of PBS was added to well 2 as a control. The Boerner slide was placed on a Venereal Disease Research Laboratory rotary shaker for 5 min and immediately read on a microtiter reading mirror. The agglutination reactions were graded as follows: 0, no agglutination; 1+, many fine clumps; 2+, a few moderate-size clumps; 3, many moderate-size clumps; and 4+, one or two large clumps. If no autoagglutination was observed in the PBS control well and a 1+ to 4+ agglutination occurred in the test well, the test was considered positive for coagulase-negative staphylococci. If no agglutination occurred, the organism was tested by a second step. In step 2, one drop of the bacterial suspension was placed into each of two wells in a Boerner slide; one drop of crude *L. polyphemus* lectin (1 mg/ml) was added to well 1, and one drop of PBS was added to well 2 as a control. The Boerner slide was placed on the shaker for 10 min and read immediately. If no autoagglutination occurred in the control well and a 1+ to 4+ agglutination occurred in the test well, the test was considered positive for coagulase-negative staphylococci. When *M. indica* extract was used as an agglutinin, the hemagglutinating activity was carefully standardized. The extract (at 1.0 mg/ml) was serially diluted and incubated in microtiter plates with an equal volume of washed type O erythrocytes in PBS. Batches of *M. indica* which gave positive agglutinations at a final extract concentration of 3.9 μg/ml were used in the studies with staphylococci. Less potent preparations were discarded.

RESULTS

Three reference American Type Culture Collection *Staphylococcus* strains and several clinically isolated laboratory strains were screened for agglutination in the rapid slide test by a battery of 14 lectins and extracts showing lectin-like activity. Of the reference strains, *S. aureus* ATCC 25923 was agglutinated by concanavalin A, and *S. epidermidis* ATCC 14990 and *S. saprophyticus* ATCC 13518 were agglutinated by *M. indica* extract. Some of the laboratory strains tested showed agglutination with *B. simplicifolia*, crude *L. polyphemus*, and WGA, as well as with *M. indica* and concanavalin A. The number of strains of each species of *Staphylococcus* tested and the patterns of agglutination obtained with these five lectins and extracts are shown in Table 1. The other nine lectins (Table 1, footnote *b*) in the screening battery were nonreactive with any of the staphylococci examined.

Three of the agglutinins selectively agglutinated *S. epidermidis* and other coagulase-negative staphylococci, whereas *S. aureus* was not agglutinated except for *S. aureus* Wood 46, which was agglutinated by WGA. The three agglutinins which were reactive only with coagulase-negative staphylococci were *M. indica* extract, crude *L. polyphemus* lectin, and WGA. *M. indica* extract was the most reactive, agglutinating 57% (29 of 51) of the coagulase-negative strains. The 22 strains which were not agglutinated with *M. indica* extract were tested for agglutination by the other two lectins which were nonreactive with *S. aureus*. WGA was reactive with 3 coagulase-negative strains, and crude *L. polyphemus* lectin agglutinated 16 coagulase-negative strains although these strains were nonreactive with *M. indica* extract. One strain developed autoagglutination in PBS upon subculture and could not be tested with crude *L. polyphemus* lectin. Two (4%) of the coagulase-negative strains did not agglutinate with any of these three agglutinins.

The majority of the coagulase-negative clinical isolates were *S. epidermidis* as determined by the criteria of the Subcommittee on Taxonomy of Staphylococci and Micrococci (38) and

TABLE 1. Comparison of *Staphylococcus* strains agglutinated by lectins or extracts[a]

Source of lectin or extract[b]	No. of strains	Organism	No. of strains:	
			Agglutinated	Not agglutinated
B. simplicifolia	12	*S. aureus*	2	10
	13	*S. epidermidis*	1	12
	1	*Staphylococcus* spp.[c]	0	1
Canavalia ensiformis[d]	18	*S. aureus*	12	6
	21	*S. epidermidis*	19	2
	5	*Staphylococcus* spp.	2	3
L. polyphemus (crude)[d]	14	*S. aureus*	0	14
	19	*S. epidermidis*	15	4
	2	*Staphylococcus* spp.	1	1
M. indica	40	*S. aureus*	0	40
	42	*S. epidermidis*	23	19[e]
	10	*Staphylococcus* spp.	7	3
T. vulgaris	32	*S. aureus*	1[f]	31
	25	*S. epidermidis*	4[g]	21
	6	*Staphylococcus* spp.	1	5

[a] One drop of the lectin or extract was added to one drop of bacterial suspension in a Boerner slide well and mixed on a rotary shaker.

[b] The following lectins or extracts were nonreactive with all of the staphylococcal strains tested: *Arachis hypogaea, Diospyros* spp., *Dolichos biflorus, Glycine max, L. culinaris, L. polyphemus* (pure), *Lotus tetragonolobus, Ricinus communis* I, and *Ulex europaeus* I.

[c] Other coagulase-negative staphylococci.

[d] Lectin and bacteria were suspended in PBS containing 1.0 mM Ca^{2+} and Mn^{2+}.

[e] The 19 strains which were nonreactive with *M. indica* extract were also tested for agglutination with *L. polyphemus* agglutinin.

[f] Strain Wood 46.

[g] Three of the four strains were not agglutinated with either *L. polyphemus* or *M. indica* agglutinin.

the schema of Kloos and Schleifer (16) (Table 2). The other coagulase-negative strains were collectively referred to as *Staphylococcus* spp. The characteristics of the biotypes of the clinical strains are listed in Table 2. All of the *S. aureus* strains were typical except that one strain was sucrose negative.

The minimum concentration of *M. indica* extract and WGA required for maximal agglutination of coagulase-negative staphylococci was 125 μg of each agglutinin per ml. This concentration was determined by testing various proportions of the combined agglutinins with *S. epidermidis* ATCC 14990, a battery of other coagulase-negative staphylococci, and *S. aureus* ATCC 25923. It was possible to combine these two agglutinins for increased specificity. All *S. epidermidis* strains were agglutinated by the combined agglutinins at a concentration of 125 μg/ml, whereas *S. aureus* ATCC 25923 was not agglutinated. The crude *L. polyphemus* agglutinin was supplied in solution at a concentration of 1 mg/ml. This was the only concentration which demonstrated reactivity in the rapid slide test. No agglutination was obtained at a concentration of 660, 500, or 250 μg/ml. Surprisingly, no agglutination was observed with purified *L. polyphemus* agglutinin at either 500 or 100 μg/ml.

These results led to the adoption of a two-step rapid slide test for the differentiation of human strains of coagulase-positive *S. aureus* and coagulase-negative staphylococci (Fig. 1). The results obtained with the two-step agglutination test for strains of each biotype are presented in Table 2. The two-step test, first with *M. indica* extract-WGA and then with crude *L. polyphemus* agglutinin, correctly differentiated 100% of the *S. aureus* strains (excluding strain Wood 46) and 96% of the coagulase-negative strains. The test is rapid, with the majority of the coagulase-negative staphylococci agglutinating within the first 5 min of shaking. When the second step is required, the total test time is no more than 15 min.

As a further check on the reliability of the test, a blind study was conducted. Fresh clinical isolates were obtained from a local hospital. After the strains were Gram stained and checked for catalase production, they were subcultured and tested for coagulase production by one researcher. The "unknown" strains were then tested in the two-step agglutination test by another researcher, and the results were

TABLE 2. *Micrococcaceae* strains tested

No. of strains	Organism	Characteristics[a]	Agglutinated[b] by:	
			M. indica-WGA[c]	Crude *L. polyphemus*
38	*S. aureus*	coag+, pho+, ana mtl+, glc+, mtl+, suc+	0/38	0/14
1	*S. aureus*	coag+, pho+, ana mtl+, glc+, mtl+, suc−	0/1	0/1
40	*S. epidermidis*	coag−, pho+, ana mtl−, glc+, mtl−, suc+, man+, mal+, tre−	25/40	14/15
2	*S. epidermidis*	coag−, pho−, ana mtl−, glc+, mtl−, suc+, man+, mal+, tre−	1/2	1/1
2	*Staphylococcus* spp.[d]	coag−, pho−, ana mtl−, glc+, mtl−, suc+, man+, mal+, tre+	0/2	0/1[e]
3	*Staphylococcus* spp.	coag−, pho−, ana mtl−, glc+, mtl+, suc+, man−, mal+, tre+	3/3	0/0
1	*Staphylococcus* spp.	coag−, pho−, ana mtl−, glc+, mtl+, suc+, man−, mal+, tre−	1/1	0/0
1	*Staphylococcus* spp.	coag−, pho−, ana mtl−, glc+, mtl+, suc+, man+, mal+, tre−	1/1	0/0
1	*Staphylococcus* spp.	coag−, pho−, ana mtl−, glc+, mtl−, suc+, man−, mal+, tre+	1/1	0/0
1	*Staphylococcus* spp.	coag−, pho+, ana mtl−, glc+, mtl−, suc−, man+, mal−, tre+	0/1	1/1
1	*Micrococcus* spp.	coag−, pho−, ana mtl−, glc−, mtl−, suc−, man−, mal−	0/1	0/1
1	*S. aureus*	ATCC 25923	0/1	0/1
1	*S. aureus*	ATCC 25904	0/1	0/1
1	*S. saprophyticus*	ATCC 13518	1/1	0/0
1	*S. aureus*[f]	Smith compact	0/1	0/1
1	*S. aureus*[f]	Wood 46	1/1	0/0
1	*S. aureus*[f]	M	0/1	0/1

[a] Abbreviations: coag, free coagulase; pho, phosphatase; ana mtl, anaerobic mannitol; glc, glucose fermentation; mtl, acid from mannitol; suc, acid from sucrose; man, acid from mannose; mal, acid from maltose; tre, acid from trehalose.

[b] Number agglutinated/number tested.

[c] A total of 125 μg of each per ml.

[d] *Staphylococcus* spp. refers to coagulase-negative staphylococcal isolates with biochemical characteristics different from those of *S. epidermidis*. For example, production of acid from trehalose or mannitol is not characteristic of *S. epidermidis* (38).

[e] One strain developed autoagglutination upon subculture and could not be tested with *L. polyphemus* lectin.

[f] Courtesy of B. Wilkinson, Illinois State University. Other strains were obtained from the American Type Culture Collection or a clinical laboratory.

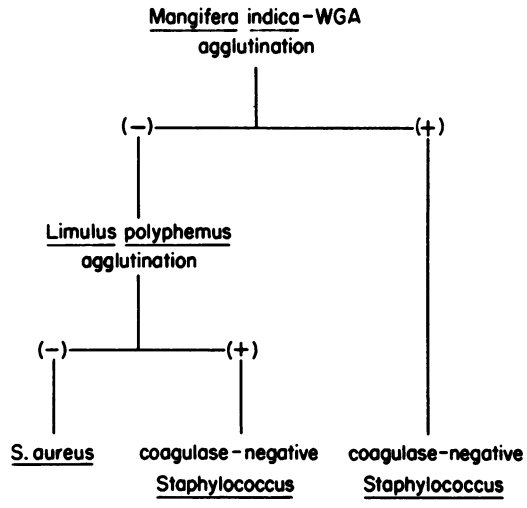

FIG. 1. Differentiation of staphylococci by agglutination procedures.

compared. The agglutination slide test correctly differentiated the 29 presumed *S. aureus* strains and the 30 strains of coagulase-negative staphylococci. In the course of this study, one strain of coagulase-negative staphylococci was detected which required 250 μg of *M. indica* extract per ml for agglutination rather than the usual 125 μg/ml. This suggested that the optimum concentration of agglutinins for the test should be 250 rather than 125 μg/ml.

DISCUSSION

Staphylococci are most commonly identified by the coagulase test, a method which has been qualitatively correlated with pathogenicity (9, 15). This conventional means of identification requires from 4 to 16 h of incubation. We have proposed a rapid, two-step agglutination slide test for the differentation of coagulase-negative and coagulase-positive staphylococci. The two-step slide test was completed in 5 to 15 min. Coagulase-negative staphylococci were agglutinated with the extract from *M. indica* plus WGA (125 μg of each per ml) and crude *L. polyphemus* lectin (1 mg/ml). *S. aureus* was not agglutinated, except for *S. aureus* Wood 46, which showed a 1+ agglutination with WGA (125 μg/ml). This reaction may have been due to the atypical cell surface of strain Wood 46. The unusual nature of the cell surface of this strain is demonstrated by the presence of small quantities of protein A (10) and of only β-linked *N*-acetylglucosamine ribitol teichoic acid (6). The cell wall of a typical *S. aureus* strain contains larger quantities of protein A (5, 20) and possesses both α- and β-linked *N*-acetylglucosamine ribitol teichoic acid (6) bound to the peptidoglycan. Possibly the absence of large quantities of protein A leaves the β-linked residues exposed for combination with WGA, which has a specificity for β-linked *N*-acetyglucosamine (3).

Two coagulase-negative staphylococci were nonreactive with the test agglutinins. These strains were subcultured for 1 to 5 months before being tested with crude *L. polyphemus* lectin and may have undergone surface modification. Antigen variation has been reported to occur in *S. aureus* (27). Some of the subcultured *S. aureus* strains developed autoagglutination in PBS, and the tests with *L. polyphemus* lectin could not be interpreted. The two-step agglutination slide test correctly differentiated 96% of the coagulase-negative staphylococci and 100% of the *S. aureus* strains (excluding strain Wood 46) tested in the initial survey of clinical isolates (Tables 1 and 2) and all 59 recent clinical isolates tested in the blind study.

Non-agglutination of *S. aureus* in the slide test did not appear to be directly related to coagulase production as measured by the tube coagulase test. *S. aureus* ATCC 25904 produces only bound coagulase, not free coagulase, and was not agglutinable (Table 2). This strain of *S. aureus* would be incorrectly identified by the tube coagulase test, which detects only free coagulase.

The agglutination patterns obtained with each of the test agglutinins were examined for correlation with eight biochemical characteristics in addition to coagulase. The strains were grouped according to similar biochemical traits and identified as *S. epidermidis* and *Staphylococcus* spp. (Table 2). *S. saprophyticus* was not characterized and would therefore be included with the *Staphylococcus* spp. Preliminary screening showed that *M. indica* extract reacted with both *S. epidermidis* ATCC 14990 and *S. saprophyticus* ATCC 13518. A homogeneous group of coagulase-negative staphylococci which agglutinated with *M. indica* extract, WGA, or *L. polyphemus* lectin was not demonstrated. *M. indica* extract agglutinated the majority of the *S. epidermidis* and *Staphylococcus* spp. strains. The three strains which agglutinated only with WGA were all *S. epidermidis*. *L. polyphemus* lectin (crude) agglutinated *S. epidermidis* strains and one sucrose-negative *Staphylococcus* sp. strain. The strains which did and did not agglutinate with *M. indica* extract-WGA did not correlate with either the biotype scheme of Baird-Parker (2) or the nine species of coagulase-negative *Staphylococcus* proposed by Kloos and Schleifer (16).

The specific nature of the reaction of the agglutinins with the bacteria was not determined. However, as pure *L. polyphemus* lectin did not agglutinate coagulase-negative staphylo-

cocci, it is assumed that the sialic acid-binding lectin present in the crude *L. polyphemus* agglutinin was not involved in the reaction. The presence of multiple lectins in crude *Limulus* preparations has been suggested (4, 12, 29), although only the sialic acid-choline phosphate-specific protein has been purified (32). The *M. indica* agglutinin appears to be nonprotein and is nonspecific in its ability to agglutinate human or animal erythrocytes (unpublished data). In this respect, the *M. indica* agglutinin is similar to that reported for *Persea americana*, a nonprotein, nonspecific agglutinin (25).

The advantages of the agglutination test over the conventional tube coagulase test are: (i) the agglutination test provides a more rapid method for identifying the clinically significant staphylococci, i.e., 5 min versus the extensive incubation time required for the tube coagulase test; and (ii) because the agglutination technique has been shown to detect *S. aureus* even when the tube coagulase test is negative, it may prove to be a more sensitive test for the recognition of *S. aureus*. The testing of large numbers of strains in clinical laboratories will be required to confirm this possibility.

In terms of routine clinical use, one limitation is the unavailability through a commerical source of *M. indica* extract. Although the test does not identify coagulase-negative staphylococci as to species, this does not pose a problem as many clinical laboratories do not so identify coagulase-negative staphylococcal isolates.

ACKNOWLEDGMENTS

This investigation was supported in part by Research Contract DAMD 17-81-C-1028 from the U.S. Army Medical Research and Development Command, Fort Detrick, Md.

We thank B. J. Wilkinson for supplying several strains.

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