AD-A151 603 RAPID METHODS FOR THE LABORATORY IDENTIFICATION OF PATHOGENIC MICROORGANISMS(U) LOUISVILLE UNIV KY R J DOYLE ET AL. SEP 81 DAMD17-81-C-1028 1/1

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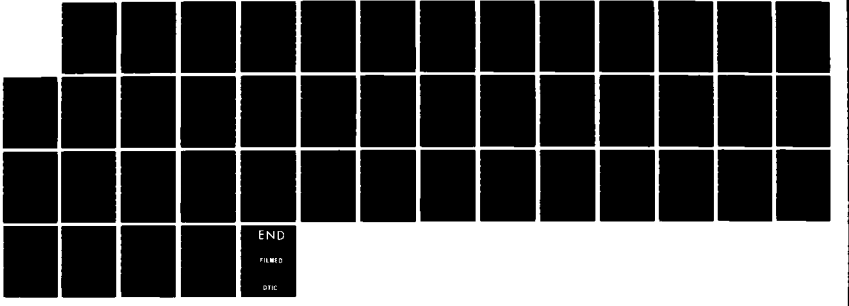

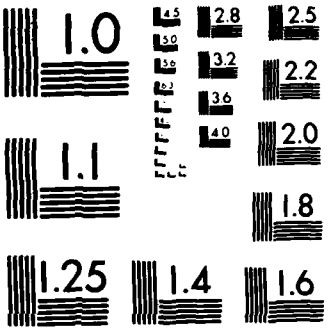

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RAPID METHODS FOR THE LABORATORY IDENTIFICATION OF PATHOGENIC MICROORGANISMS

ANNUAL REPORT

Ronald J. Doyle, Ph.D.
Kenneth F. Keller, Ph.D.

September 1981

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-81-C-1028

University of Louisville
Louisville, Kentucky 40292

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A151 603	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Rapid Methods for the Laboratory Identification of Pathogenic Microorganisms		5. TYPE OF REPORT & PERIOD COVERED Annual Report (1 Feb 81 - 31 Jul 81)
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Ronald J. Doyle, Ph.D. Kenneth F. Keller, Ph.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD17-81-C-1028
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of Louisville Louisville, Kentucky 40292		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS10.AN.058
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701-5012		12. REPORT DATE September 1981
		13. NUMBER OF PAGES 44
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

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PROGRESS REPORT

Work on this research began 1 February, 1981. Progress recorded on the following pages constitutes results obtained through July, 1981, or six months.

In accordance with our first year objectives and desires of U.S. Army personnel at Ft. Detrick, we pursued studies which would validate our proposal that lectins could be used to "type" or identify bacteria. The results are arranged in the form of manuscripts or in the form of tables with accompanying comments. The results are promising and tend to suggest that lectins could be used to diagnose some microbial agents of military concern. The Principal Investigator also spent some time developing a diagnostic test for deep-seated Staphylococcus aureus infections. This latter work does not have priority but is considered within the realm of interest of the U.S. military. A recently submitted manuscript is enclosed as part of the Progress Report.

Our first six month progress can be summarized along the following lines:

a. Coagulase-negative and coagulase-positive staphylococci can be differentiated on the basis of a simple and rapid lectin agglutination slide test. The manuscript describes the test and results. A tentative acceptance from the J. Clin. Microbiol. has been received.

b. Many serotypes of Legionella and several Legionella-like organisms can be rapidly differentiated by agglutination reactions with plant agglutinins. Positive agglutinations are dependent on the method of culturing the organisms (table enclosed) but are independent of animal passage of the bacteria. Furthermore, heat treatment of the cells modifies their reactivities with the agglutinins.

c. Poly(glycerolphosphate) is a common antigen for many Gram-positive bacteria and may interfere with the commonly employed serological tests for Staphylococcus aureus antibodies.

d. Preliminary results suggest that the most common pathogenic streptococci can be "typed" using lectins.

e. Preliminary results provide strong evidence to show that the fungi, Candida and Cryptococcus, can be rapidly differentiated by a lectin test.

LECTINS IN DIAGNOSTIC MICROBIOLOGY. DIFFERENTIATION OF COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI BY LECTINS AND PLANT AGGLUTININS

Sandra K. Davidson, Kenneth F. Keller*, and Ronald J. Doyle

Department of Microbiology and Immunology
University of Louisville School of Medicine
Louisville, Kentucky 40292

Running title: Lectin - Staphylococcus Interaction

*Address correspondence to:

K.F. Keller
Department of Microbiology
and Immunology
University of Louisville
School of Medicine
Louisville, Kentucky 40292

ABSTRACT

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 identification of coagulase-negative and coagulase-positive staphylococci. The coagulase-negative staphylococci were agglutinated by agglutinins from *Mangifera indica*, *Triticum vulgaris*, and *Limulus polyphemus*. The test is rapid, requiring only 5 to 15 min to identify an unknown staphylococcus, as opposed to 4-16 hrs required to perform the conventional tube coagulase test.

INTRODUCTION

For species identification of staphylococci, the ICSB Subcommittee on Taxonomy of Staphylococci and Micrococci recommend 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 the research laboratory. In the clinical laboratory, staphylococcal isolates are most commonly identified by the tube coagulase test which detects "free" coagulase (9). This test is often difficult to interpret and false-negative (36,40) and false-positive (2,36,40) tests have been reported.

Lectins are proteins or glycoproteins of non-immune origin with sugar-specificity (13,22,35). Some plant extracts also contain substances which may agglutinate several types of red cells (25). By a strict definition, these "agglutinins" are not lectins because they do not possess carbohydrate specificities and are not proteins (25). 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 ten years, some researchers have demonstrated the ability of lectins to agglutinate certain species of bacteria (12,18,19,21,24,26,28,30,39). The specificity of agglutination of bacteria by lectins resides in the unique cell surface structures of the bacteria interacting with the carbohydrate-specific lectins (1,17,19,26,29,31,33). Only recently has lectin agglutination of bacteria been utilized as a method for the definitive identification of clinical isolates. Wheat germ agglutinin (Triticum vulgaris) has been shown to

specifically agglutinate Neisseria gonorrhoeae and not to agglutinate encapsulated Neisseria meningitidis (13,33,34).

The objective of these studies was to develop a rapid identification procedure for staphylococci using lectins or plant agglutinins. Reference strains of staphylococci, as well as 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 employing Mangifera indica (mango) extract, wheat germ agglutinin (WGA), and crude Limulus polyphemus (horseshoe crab) lectin was found to agglutinate 96% (79 of 82) of the coagulase-negative strains of staphylococci and only 1% (1 of 73) of the Staphylococcus aureus strains.

MATERIALS AND METHODS

Reagents. All chemicals, salts, and sugars were of the highest analytical grade available. Routinely used lectins were purchased from E-Y Laboratory (San Mateo, CA). M. indica extract was prepared from the dried seeds of commercially purchased mangoes. The seeds were pulverized in a CRC micromill and extracted for 2 hr at room temperature in 10 times their weight of PBS (0.05M potassium phosphate, 0.15M sodium chloride, pH 7.2). Insoluble material was removed by centrifugation and the supernatant dialyzed against 5 liters of distilled water. The crude M. indica extract was centrifuged and lyophilized. The Mangifera extract agglutinated various types of red cells (unpublished observations). Characterization and biological properties of the agglutinin activity will be reported elsewhere (Davidson, Doyle, Keller, in preparation). Partially purified Lens culinaris (commercial lentil), Diospyros sp. (commercial persimmon), and Bandeiraea simplicifolia (Calbiochem, San Diego, CA) 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 freeze-dried and tested for hemagglutinating activity.

Microorganisms. Reference strains were S. aureus ATCC strains 25923 and 25904, Staphylococcus epidermidis ATCC strain 14990, and Staphylococcus saprophyticus ATCC strain 13518. Other microorganisms tested included 39 clinical isolates of S. aureus, 44 strains of S. epidermidis, 7 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. The clinically isolated microorganisms were identified by Gram stain, catalase

production, glucose fermentation (8), the oxidation and fermentation of mannitol (2%, wt/vol) in tubes of cysteine-trypticase agar medium (BBL Microbiology Systems, Cockeysville, MD), coagulase production (37), acid production from 1% (wt/vol) maltose, mannose, sucrose, and trehalose in purple broth base (Difco Laboratories, Inc., Detroit, MI) (14), and phosphatase production (17).

Agglutination slide test for the identification of coagulase-positive and coagulase-negative staphylococci. The agglutination slide test was performed according to 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 of each agglutinin was 125 µg/ml. One drop of the bacterial suspension was placed into each of two wells in a Boerner slide; to well no. 1, one drop of the M. indica extract-WGA solution was added, and to well no. 2 (control), a drop of PBS was added. The Boerner slide was placed on a Venereal Disease Research Laboratory (VDRL) 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+=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 in step two. In step two, one drop of the bacterial suspension was placed into each of two wells in a Boerner slide; to well no. 1, one drop of crude L. polyphemus lectin (1 mg/ml) was added, and to well no. 2 (control), a drop of PBS was added. The Boerner

slide was placed on the VDRL 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 Mangifera extract was used as an agglutinin, the hemagglutinating activity was carefully standardized. The extract (at 1.0 mg/ml) was serially diluted and interacted with an equal volume of human type O erythrocytes in microtiter plates. Batches of Mangifera which gave positive agglutinations at a final extract concentration of 3.9 µg/ml were used in studies with staphylococci. Less potent preparations were discarded.

RESULTS

Reference ATCC Staphylococcus strains and clinically-isolated laboratory strains were screened for agglutination in the rapid slide test by a battery of fourteen lectins and extracts containing lectin-like activity. Only concanavalin A and M. indica extract agglutinated some of the reference strains (Table 1). Some of the laboratory strains tested showed agglutination with Bandeiraea simplicifolia, crude L. polyphemus, and WGA, as well as with M. indica and concanavalin A. The numbers of strains of each species of Staphylococcus tested and the patterns of agglutination obtained with these five lectins (agglutinins) are presented in Table 2. The other nine lectins in the screening battery were non-reactive with all of the staphylococci examined (data not shown).

Three of the agglutinins selectively agglutinated S. epidermidis and other coagulase-negative staphylococci, whereas, S. aureus was not agglutinated, except for S. aureus strain Wood 46, which was agglutinated by WGA. The three agglutinins which were reactive only with coagulase-negative staphylococci were M. indica, crude L. polyphemus, and WGA. M. indica extract was the most reactive, agglutinating 57% (29 of 51) of the coagulase-negative strains. All of the strains which were not agglutinated with M. indica were tested for agglutination by the other two lectins which were unreactive with S. aureus. Wheat germ agglutinin was reactive with three coagulase-negative strains, and crude L. polyphemus lectin agglutinated 16 coagulase-negative strains, although these strains were unreactive 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 ICSB Subcommittee on Taxonomy of Staphylococci and Micrococci (38), and the schema of Kloos and Schleifer (16) (Table 3). 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 3. 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/ml of each agglutinin. This was determined by testing combinations of dilutions of both agglutinins with S. epidermidis ATCC 14990, a battery of other coagulase-negative staphylococci, and S. aureus ATCC 25923 (Table 4). It was possible to combine these two agglutinins for increased specificity. 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 concentrations of 660, 500 or 250 µg/ml. Surprisingly, no agglutination was observed with purified L. polyphemus agglutinin (at 500 and 100 µg/ml).

The foregoing results led to the adoption of a two-step rapid slide test for the identification 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 3. The two-step test, using M. indica extract-WGA, then crude L. polyphemus agglutinin, correctly identified 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 compared (Table 5). The agglutination slide test correctly identified 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/ml of M. indica extract for agglutination rather than the usual 125 µg/ml. This suggested that the optimum concentration of agglutinins for the test should be 250 µg/ml rather than 125 µg/ml.

DISCUSSION

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

Two coagulase-negative staphylococci were unreactive with the test agglutinins. These strains were subcultured for 1 to 5 months before being tested with crude L. polyphemus 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 could not be interpreted. The two-step agglutination slide test correctly identified 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 2 and 3) and all 59 recent clinical isolates tested in the blind study (Table 5).

Non-agglutination of S. aureus in the slide test did not appear to be directly related to coagulase production. S. aureus ATCC 25904 produces only bound coagulase, not free coagulase, and was not agglutinable (Table 3). 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 3). S. saprophyticus was not speciated and would therefore be included with the Staphylococcus spp. Preliminary screening (Table 1) 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 S. epidermidis. L. polyphemus (crude) agglutinated S. epidermidis strains and one sucrose-negative Staphylococcus spp. strain. The M. indica extract-WGA agglutinable and non-agglutinable strains did not correlate with the biotype scheme of Baird-Parker (2) or with the seven 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 staphylococci, it is assumed that the sialic acid-binding lectin present in the crude Limulus was not involved in the reaction. The presence of multiple lectins in crude Limulus has been suggested (4,12,29), although only the sialic acid-choline phosphate specific protein has been purified (32). The Mangifera agglutinin appears to be non-protein and is non-specific in its ability to selectively agglutinate human or animal red cells (unpublished observations). In this respect, the Mangifera agglutinin is similar to that reported for Persea americana, a non-protein, non-specific 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. Five min vs the extensive incubation time required for the tube coagulase test. ii) Because the agglutination technique has been shown to detect S. aureus, even when the tube coagulase was negative, it may prove to be a more sensitive test for pathogenicity. The testing of large numbers of strains in the clinical laboratory will be required in order to confirm this possibility.

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 several strains.

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Table 1. Agglutination of Staphylococcus species by lectins and plant agglutinins

LECTINS OR EXTRACTS	REACTIONS[a]		
	S. aureus ATCC 25923	S. epidermidis ATCC 14990	S. saprophyticus ATCC 13518
Arachis hypogaea	-	-	-
Bandeiraea simplicifolia	-	-	-
Canavalia ensiformis[b]	+	-	-
Diospyros spp.	-	-	-
Dolichos biflorus	-	-	-
Glycine max[b]	-	-	-
Lens culinaris[b]	-	-	-
Limulus polyphemus (crude)[b]	-	-	-
Limulus polyphemus (pure)[b]	-	-	-
Lotus tetragonolobus	-	-	-
Mangifera indica	-	+	+
Ricinus communis I	-	-	-
Triticum vulgaris	-	-	-
Ulex europaeus I	-	-	-

[a] One drop of lectin (125 µg/ml to 1mg/ml) was added to one drop of bacterial suspension in a Boerner slide well and mixed on a rotary shaker.

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

Table 2. Comparison of *Staphylococcus* strains agglutinated by lectins or extracts[a]

Lectins or extracts	No. of strains	Organisms	Agglutinated (No. of strains)	Not agglutinated (No. of strains)
Bandieraea simplicifolia	12	*S. aureus*	2	10
	13	*S. epidermidis*	1	12
	1	*Staphylococcus spp.*[b]	0	1
Canavalia ensiformis	18	*S. aureus*	12	6
	23	*S. epidermidis*	19	4
	3	*Staphylococcus spp.*	2	1
Limulus polyphemus (crude)	14	*S. aureus*	0	14
	20[c]	*S. epidermidis*	15	5
	1	*Staphylococcus spp.*	1	0
Mangifera indica	40	*S. aureus*	0	40
	44	*S. epidermidis*	23	21[c]
	7	*Staphylococcus spp.*	6	1
Triticum vulgaris	32	*S. aureus*	1[d]	31
	27	*S. epidermidis*	4[e]	23
	5	*Staphylococcus spp.*	1	4

Table 2. continued

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

[b]Other coagulase-negative staphylococci.

[c]Same strains.

[d]Strain Wood 46.

[e]3 of 4 strains not agglutinated with *Limulus* or *Mangifera* agglutinins.

Table 3. Micrococcaceae strains tested[a]

No. of strains	Organisms	Characteristics	*Mangifera*-WGA agglutination[d]	*Limulus* agglutination[e]
38	*S. aureus*	coag+,pho+,ana mtl+,glc+,mtl+,suc+	0/38[g]	0/14[g]
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.[b]	coag-,pho-,ana mtl-,glc+,mtl-,suc+,man+,mal+,tre+	0/2	0/1[f]
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	0/1
1	*S. aureus*[c]	Smith compact	0/1	0/1
1	*S. aureus*[c]	Wood	1/1	0/0
1	*S. aureus*[c]	M	0/1	0/1

Table 3. continued

[a] Abbreviations used: 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] Other coagulase-negative staphylococci.

[c] Courtesy of B. Wilkinson, Illinois State University. Other strains obtained from ATCC or clinical laboratory.

[d] 125 µg/ml of each.

[e] Crude L. polyphemus lectin.

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

[g] Data values indicate number positive over number tested.

Table 4. Slide agglutination results with dilutions of combined *Mangifera* and wheat germ agglutinins

STRAINS	SLIDE AGGLUTINATION RESULTS[a]			
	0.5mg/ml[b]	0.25mg/ml	0.125mg/ml	0.062mg/ml
S. epidermidis				
ATCC 14990	3+	2+	2+	0
83	2+	2+	2+	2+
86	3+	2+	2+	2+
92	2+	2+	2+	2+
94	2+	2+	2+	2+
S. aureus ATCC 25923	0	0	0	0

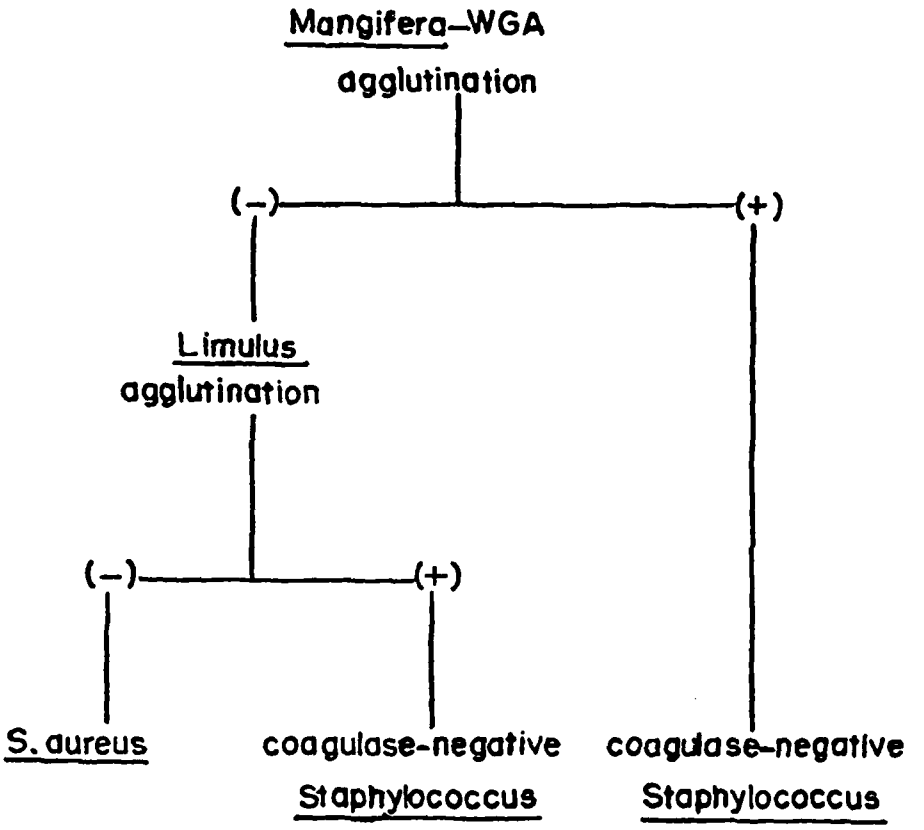
[a] One drop of combined agglutinins was added to one drop of bacterial suspension in a Boerner slide and mixed for 5 min on a rotary shaker.

[b] Mg/ml of each agglutinin prior to mixing with cells.

Table 5. Blind study results of lectin agglutination test versus tube coagulase test

	No. of strains identified by lectin agglutination	Percent confirmed by tube coagulase[a]
Staphylococcus aureus	29	100%
Coagulase-negative staphylococci	30	100%

[a] Performed by another researcher.


Mangifera–WGA
agglutination
(–)
(+)
Limulus
agglutination
(–)
(+)
S. aureus
coagulase-negative
Staphylococcus
coagulase-negative
Staphylococcus

Legends for Figure.

Fig. 1. Proposed diagnostic agglutination test.

Anti-Polyglycerolphosphate in Human Sera

Ronald J. Doyle,*[1] Elaine M. Sonnenfeld,[2] Karen Cost[2],

and Suthep Thaniyavarn[1]

Department of Microbiology and Immunology
University of Louisville[1]
Health Sciences Center
Louisville, KY 40292

and

Norton-Children's Hospital[2]
Louisville, KY 40202

Running title: anti-lipoteichoic acid

*Address correspondence to:

R.J. Doyle
Department of Microbiology and Immunology
University of Louisville
School of Medicine
Louisville, KY. 40292

(502) 588-5350

Ultrasonic extracts from staphylococci contain red cell-sensitizing poly(glycerolphosphate)teichoic acid antigen. The antigen may contribute to the variability of precipitin reactions used in the diagnosis of Staphylococcus aureus infections.

The diagnosis of deep-seated staphylococcal infections often involves the use of serological techniques. Because Staphylococcus aureus presents many antigenic determinants, including the immunoglobulin-reactive protein A, it has been difficult to establish specific and quantitative methods to accurately analyze a patients serum for anti-staphylococcal antibodies. One commonly employed method, designed to avoid interference from protein A, involves the measurement of antibodies directed against teichoic acid (4-7,8,10,11). The method uses sonicated suspensions of protein A-deficient S. aureus cells as antigens in counterimmunoelectrophoresis assays (5,10). Serum titers as low as 1:4 and 1:8 have been accepted as presumptive evidence for a S. aureus infection (8,11). Wheat and White (11) have recently reviewed the methods for the serological diagnosis of staphylococcal infections.

We observed that a trypsinized, cell wall teichoic acid-deficient mutant of S. aureus (strain 52A5, see ref. 7 for a description of its characteristics) was readily agglutinable by a rabbit antiserum against a poly(glycerolphosphate) teichoic acid. Hamada et al. (2) showed that hot saline extracts from various Staphylococcus species were reactive with anti-poly(glycerolphosphate). Recently, Aasjord and Grov (1) provided evidence to suggest that poly(glycerolphosphate) was a surface antigen for S. aureus, even though the poly(glycerolphosphate) (PGP) was not a cell

wall component. These results suggested that the membrane-bound lipoteichoic acid (LTA), which contains repeating glycerol phosphate residues could contribute to the reactivity of S. aureus antigen preparations with a patient's serum.

A standard method for the preparation of teichoic acid antigen for use in counter-immunoelectrophoresis assays is to sonicate suspensions of S. aureus and standardize the ultrasonic extract on the basis of protein concentration (4,5,10). The protein content presumably reflects the extent of cell breakage and may not be a reliable indicator for wall teichoic acid, although this will not be addressed in this paper. When we prepared ultrasonic extracts from several different strains of Staphylococcus, we found that the extracts did indeed contain poly(glycerolphosphate) antigen (Table 1). The assay was based on the red cell sensitizing properties (3) of lipoteichoic acids. The ultrasonic extracts were mixed with washed red cells and the sensitized cells mixed with dilutions of a rabbit antiserum against Bacillus subtilis gtaB lipoteichoic acid (9). In every case, the red cells absorbed antigens from the ultrasonic extracts which reacted with the specific LTA antiserum resulting in hemagglutination (Table 1). When purified B. subtilis gtaB cell wall teichoic acid (9) was added to the sensitized cells prior to interaction with the antisera, complete inhibition of hemagglutination was observed. Because the cell wall teichoic acid is a poly(glycerolphosphate) (9), it follows that the hemagglutination depends on PGP- anti-PGP interactions. Moreover, when similar amounts of a cell wall teichoic acid from S. aureus H [the teichoic acid is poly(ribitol phosphate), substituted with α, or β-N-acetyl-D-glucosamine and D-alanine residues, ref. 7] were added to the sensitized red cells, no inhibition of hemagglutination was observed when the anti-PGP serum was added.

Sera from several patients were also tested for the presence of anti-poly(glycerolphosphate) by use of the passive hemagglutination method (Table 2). The results reveal that both control sera and sera from individuals who had confirmed infections due to staphylococci contained anti-PGP antibodies. In general, the individuals who had higher titers were those who had active infections. Three individuals, however, who were free of apparent infections, possessed anti-PGP titers of 1:64. We are not proposing the use of anti-PGP titers as a diagnostic method for staphylococcal infections, because PGP is a common antigen for many Gram-positive bacteria. Studies are under way to monitor changes in anti-PGP titers during the course of infections due to Gram-positive bacteria.

We believe that one reason for the variability in results obtained by the precipitin techniques (4,5,10,11) is the presence of anti-PGP in the patients serum (Table 2) and the presence of PGP in the antigen preparation (Table 1). Caution must therefore be exercised in interpretation of data which deal with staphylococcal surface antigens unless care has been made to eliminate interference by PGP-anti-PGP interactions.

ACKNOWLEDGMENTS

We thank B.J. Wilkinson for some of the staphylococcal strains. This work was supported in part by U.S. Army Contract DAMD17 81C 1028.

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Table 1. Release of red cell sensitizing poly(glycerolphosphate) from suspensions of staphylococcal cells[a].

Organism	Protein (µg/ml)	Absorbance (500nm)	LTA Level
S. aureus Smith (diffuse)	55	0.11	1:256
S. aureus Wood 46	30	0.13	1:32
S. aureus M	41	0.16	1:32
S. aureus M variant	38	0.12	1:32
S. aureus ATCC 11587	27	0.11	1:256
S. epidermidis	8	0.07	1:16

[a] Staphylococci were washed twice in 0.15M sodium chloride, 50mM sodium phosphate (pH 7.4) and finally suspended to a density of 1.0 absorbance units (500nm, 1-cm). The suspensions were then subjected to single 5 min sonic treatments (Heat Systems-Ultrasonics, Inc., Model W140, Plainview, NY) at 1-3°C. The disrupted cells were centrifuged at 20,000 x g for 10 min. The supernatants were then analyzed for turbidity and protein content and were used to sensitize human type O red cells. The sensitization procedure consisted of mixing 1.0 ml of washed red cells in PBS (1% packed cells) with 1.0 ml of the sonicate for 1 hr at room temperature. The sensitized cells were then washed twice with 10.0 ml volumes of PBS and finally suspended to 1.0 ml for assay. A sample of anti-poly(glycerolphosphate) (anti Bacillus subtilis gtaB290 teichoic acid, ref. 9), which had a titer of 1:1054 against a suspension of LTA-saturated red cells, was used to assay for the presence of antigens. The antigen level, not an actual titer, represents the dilution of the standard antiserum which will agglutinate the sensitized red cells.

Table 2. Anti-PGP titers in patient populations[a].

Patient	Anti-PGP Titer
Control (no known infection, 6)	< 1:2
Control (no known infection, 4)	1:4
Control (no known infection, 6)	1:8
Control (no known infection, 3)	1:64
Osteomyelitis (S. aureus, 2)	1:256
Osteomyelitis (S. aureus, 1)	1:128
Osteomyelitis (S. aureus, 2)	1:32
Wound, foot (S. aureus) (2)	1:64
Wound, knee (S. aureus) (1)	1:8
Wound, arm (S. aureus) (2)	1:128
Facial abscess (S. aureus) (2)	1:128
Meningitis (S. aureus) (1)	1:256
Meningitis (S. epidermidis) (1)	1:256

[a] Serum titers determined by passive hemagglutination of PGP-sensitized human red cells. The sensitizing agent was B. subtilis gtaB290 LTA. Sensitization of red cells was with 1.0 µg/ml LTA in a 1% red cell suspension (pH 7.4). The diagnoses were confirmed by isolation of the organisms. Numbers in parentheses refer to the number of patients tested. Sera were obtained at the height of symptoms or upon admission to the hospital.

RAPID-SLIDE AGGLUTINATION OF STREPTOCOCCAL SEROTYPES WITH LECTINS

Objective: to develop a rapid slide agglutination test for the identification of the clinically important streptococci (Groups A, B, C, D, and G).

Progress: In the initial phases of this research a battery of commercially purchased lectins, as well as plant agglutinins extracted in our laboratories, were empirically tested for their agglutinating activity with streptococcal groups A, B, C, D and G. As seen in Table I, three general kinds of agglutinating activities were demonstrated by the extracts and lectins tested thus far.

(a) those that showed little or no reactivity with any of the streptococcal serotypes.
(b) those that are non-specific in that they interact with three or more of the different serotypes, and
(c) those that demonstrate a strong reactivity with a specific serotype and only minimal agglutination with others.

Those lectins and/or extracts that appear to be the most promising in terms of serogroup specificity are shown in Table II.

Present and Future Activities:

(a) all the parameters known to affect conventional agglutination reactions are currently being examined; e.g. pH, concentration ratios of antigens to agglutinins, ionic concentrations of diluents, time, temperature, etc.

Because Group A streptococci contain a hyaluronic acid capsule we will pretreat these cells with hyaluronidase, in an attempt to enhance their agglutinability. Should this be demonstrated, it may provide a solution to solving the problem encountered with those lectins that presently show a strong Gr. A reactivith but also a minimal interaction with other serotypes. The rationale being that with an increased sensitivity for Gr. A, greater dilutions of the lectin may be employed and thus "dilute out" the minimal reactivity of other groups.

(b) after determining optimal conditions for routine performance of the test, both in terms of sensitivity and selectivity, large numbers of fresh streptococcal isolates will be tested in order to insure its reproducibility.

(c) despite the fact that we have found several lectins that show promise for distinguishing the clinically important groups of streptococci, we will still continue to randomly screen new plant extracts which may prove to be superior to those already tested.

TABLE I

RESULTS OF EMPIRICAL SCREENING OF LECTINS FOR AGGLUTINATING ACTIVITY WITH CLINICALLY IMPORTANT STREPTOCOCCI*

LECTIN OR AGGLUTININ	STREPTOCOCCUS GROUP**				
	A	B	C	D	G
Commercial Preparations					
Canavalia ensiformis (p)**	2+	2+	0	4+	0
Glycine max (p)	0	0	3+	0	auto-aggl.
Arachis hypogaea (p)	0	0	1+	0	0
Wisteria floribunda (c)	0	0	0	0	0
Abous precatorius (p)	0	4+	0	0	±
Lotus tetragonolobus (p)	0	0	4+	1+	0
Bandeiraea simplicifolina (c)	0	0	2+	0	1+
Triticum vulgaris (p)	4+	0	0	0	3+
Dolichos biflorus (c)	0	0	0	0	0
Limulus polyphemus (c)	4+	2+	2+	2+	1+
Robinia pseudoacacia (c)	0	0	0	0	0
Lens culinaris (p)	0	0	0	0	0
Ulex europaeus	0	0	0	0	0
Phyto-P	1+	±	0	0	0
Crude Plant Extracts Prepared in our Lab					
Mimosa seed	4+	0	±	0	±
Mango seed	0	0	1+	3+	±
Sunflower seed	2+	0	0	±	0
Avocado seed	1+	3+	3+	1+	NT
Persimmon seed	4+	0	2+	2+	0
Aloe plant	0	0	3+	±	±

** = (p) pure (c) crude.

* = streptoccal cells were removed from blood agar plates after 18 hrs incubation. Milky of cells were prepared in PBS (pH 7.2). One drop of lectin solution was added to one drop of cell suspension. Aggl. reactions read after shaking for 5 min.

TABLE II

LANCEFIELD GROUP	LECTINS OR EXTRACTS						
	PERSIMMON	MIMOSA	SUNFLOWER	ARBUS PRECATORIUS	GLYCINE MAX	ALOE	MANGO
A (8 strains)	+	+	+	-	-	-	0
B (4 strains)	-	-	-	+	-	-	0
C (3 strains)	-	-	-	-	+	+	0
D (6 strains)	-	-	-	-	-	-	+
G (2 strains)	-	±	-	-	-	±	+

Summation
Lectin-*Legionella* Interactions

Objective: To differentiate the serotypes of *Legionella pneumophila* and other *Legionella* species with the aid of lectins and plant agglutinins.

Progress:

The table on the following page shows that it is possible to "type" the serotypes of *Legionella pneumophila* with four plant agglutinins. For the six serotypes, the patterns of agglutination are unique. For *L. micdadei*, *L. bozemanii*, *L. dumoffii* and *L. gormanii*, it is presently impossible to fit them into a "typable" category.

Heating the cell suspensions (100°C, 15 min) results in modified reactivities with lectins.

Growth on different media may result in different reactivities with lectins.

Present and Future Studies: We will complete the table as shown but with the addition of *L. micdadei*, *L. bozemanii*, and *L. gormanii*. We will examine reactivities with lectins when the organisms have been passed through an experimental animal, such as the guinea pig. We will also examine reactivities when the cells have been treated with proteases. These studies should be complete by the end of the current funding year.

Table 1. Interactions between plant agglutinins and Legionella and Legionella-like bacteria.[a]

Bacterium	Agglutinin	Aloe arborescens				Mangifera indica				Persea americana				Albizzia julibrissin			
		CYE		GC-FC		CYE		GC-FC		CYE		GC-FC		CYE		GC-FC	
		RT	H	RT	H	RT	H	RT	H	RT	H	RT	H	RT	H	RT	H
L. pneumophila	California [1]	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-
L. pneumophila	Bloomington [3]	+	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-
L. pneumophila	Bellingham [1]	+	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-
L. pneumophila	Los Angeles [4]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. pneumophila	Pontiac [1]	+	+	+	-	-	-	+	-	+	-	+	+	-	-	-	-
L. pneumophila	Philadelphia [1]	+	+	+	-	+	-	+	-	+	+	+	-	-	-	-	-
L. pneumophila	Knoxville [1]	+	+	-	-	-	-	+	-	+	+	+	+	-	-	-	-
L. pneumophila	Chicago [6]	+	+	+	-	+	+	+	-	+	+	+	+	+	-	+	-
L. pneumophila	Tagus [2]	+	+	-	-	+	-	+	-	+	-	+	-	+	-	-	-
L. pneumophila	Dallas [5]	+	-	+	-	+	-	+	-	+	+	+	+	+	+	-	-
L. dumoffii		+	+	+	+	-	+	-	+	-	+	-	+	-	-	-	-

[a] CYE, charcoal-yeast extract; GC-FC, GC (Difco medium base) plus L-cys and ferric pyrophosphate; RT, cells were washed at room temperature; H, cells were boiled for 15 min, then washed and suspended. Numbers in brackets refer to serotypes.

SUMMATION

LECTIN-YEAST INTERACTIONS

Objective:

To find a lectin that selectively agglutinates Cryptococcus neoformans (the etiologic agent of cryptococcal meningitis) using a rapid 6 min. slide agglutination test. The development of such a test would obviate the need for secondary biochemical tests which require 24-48 hrs incubation for a definitive identification.

Progress:

Up to the present time, preliminary screening of the test organisms with 24 different lectins has been accomplished. The data derived from these random screening tests suggests that Triticum vulgaris (WGA), Arachis hypogaea (peanut), Conavalia ensiformis (Con A) and mango extract may potentially be utilized to differentiate Cryptococcus from the other yeasts most commonly isolated from clinical material. Although additional lectins and plant extracts will be tested in the future for the purpose of finding a cryptococcal-specific agglutinin, it may ultimately develop that Cryptococcus can be recognized by its nonagglutinability. As shown in Table I, Con A (1 mg/ml conc) agglutinates all the clinically important yeasts with the exception of Cryptococcus. It is interesting to note that despite the presence of a relatively large polysaccharide capsule on most strains of Cryptococcus none of the lectins tested, to date, were able to agglutinate the organism.

Ongoing Activities:

Using the lectins shown in Table 1, we are presently examining all the parameters known to affect the agglutination reaction, e.g. effects of pH, concentration ratios of antigens to agglutinin, time, temperature, physical and chemical pretreatment of the yeast cells which may enhance agglutinability. After determining the optimal tests conditions, a large number of strains of each species will be tested in order to ascertain whether antigenic variation among strains possess any problems in terms of reproducibility.

Future Studies:

In addition to the encouraging preliminary data with respect to the potential for separating the Genus Candida from Cryptococcus, there is also an indication of the feasibility of differentiating the species within the Candida genus.

With the development of a reliable lectin agglutination test, it would provide valuable basic data for attempting to identify these yeasts directly in clinical material. We propose to fluorescein label the species specific agglutinins, and use these conjugates as specific histochemical reagents for the direct staining of clinical materials, such as sputum and spinal fluid. If successful, it would offer a means by which the most common pathogic yeasts could be easily

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