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Slide Agglutination Method for the Serological Identification of *Neisseria gonorrhoeae* with Anti-Gonococcal Antibodies Adsorbed to Protein A-Containing Staphylococci

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A rapid slide agglutination test has been developed for the identification of *Neisseria gonorrhoeae* that are primarily detected as oxidase-positive colonies in gonococcal cultures. The technique is based on the specific nonimmune reactivity between the Fc portion of immunoglobulin (Ig)G and staphylococcal protein A. IgG molecules adsorbed to stabilized staphylococci will thereby become oriented with their antigen-reactive sites that are directed outwards. Protein A-containing staphylococci with unabsorbed anti-gonococcal antibodies gave positive co-agglutination reactions with gonococci but also with meningococci, some *Moraxella*, *Haemophilus*, and *Pseudomonas* strains. These cross-reactions were eliminated by absorption of the anti-gonococcal antiserum with meningococcal and *Moraxella* organisms prior to the coating of reagent staphylococci. In the routine culture diagnosis of *N. gonorrhoeae* the use of specific gonococcal reagent staphylococci gave concordant results with fermentation procedures and immunofluorescent techniques.

The identification of *Neisseria gonorrhoeae* in laboratory cultures is primarily based on the presence of oxidase-positive colonies of typical morphology. Colonies of other microorganisms may also react with the oxidase reagent. Some of these strains are easily distinguished from gonococcal colonies by an experienced examiner. Oxidase-positive colonies of some species of *Moraxella*, *Haemophilus*, *Pseudomonas*, and also *Neisseria* other than gonococci may, however, be impossible to distinguish on the basis of colony form. Confirmatory tests are therefore necessary. For the correct laboratory diagnosis of *N. gonorrhoeae*, the criteria should also include the demonstration of gram-negative diplococci and fermentation tests showing acid produced from glucose but not from maltose or levulose (18, 20). Pure cultures are usually needed for the fermentation tests which also require media of high quality (10, 18, 20). These tests are laborious and time consuming. In many laboratories the fermentation reactions have therefore been replaced by immunofluorescence tests when specimens from the genitourinary tract are analyzed (18, 20; D. Danielsson, Ph.D. thesis, Univ. of Uppsala, Sweden, 1965). In specimens from other sites, i.e., throat specimens, blood, cerebrospinal fluid or joint exudate, the identification of gonococcal-like colonies by immunofluorescence tests must be confirmed by fermentation procedures and sometimes also by other bacteriological tests (3, 4, 9, 20, 22).

Recently, a co-agglutinating serological reagent was described by Kronvall (14), and its use was exemplified in the capsule typing of pneumococci. This reagent was also utilized for the serological grouping of streptococci (5) as well as for mycobacterial typing (11). Reagent particles consist of formaldehyde and heat-treated Cowan I staphylococci which are subsequently coated with specific antibody via the gamma globulin Fc-protein A reaction. The antibody molecules will thereby become oriented with the antigen-reactive Fab parts that are directed outwards (14).

In the present paper the co-agglutination method has been adopted for the identification of *N. gonorrhoeae*. The results obtained in the routine identification of gonococci using *N. gonorrhoeae*-specific reagent are compared with the results obtained using immunofluorescence and fermentation tests.

MATERIALS AND METHODS

Gonococcal strains. Ninety strains of *N. gonorrhoeae* were studied in the experiments. Seven of them were laboratory strains representing colonial

morphology types T1 through T4 (12). Two of these were originally isolated at the Department of Clinical Bacteriology, Central County Hospital, Örebro, Sweden, and the other five were isolated at the State Serum Institute, Copenhagen, Denmark, at the Center for Disease Control, Atlanta, Ga., at the Institute of Medical Microbiology, Uppsala University, Uppsala, Sweden, and at the Department of Clinical Bacteriology, Södersjukhuset, Stockholm, Sweden. The other eighty-three strains were freshly isolated gonococcal strains, predominantly of colonial morphology types T1 or T2, obtained from routine cultures of genito-urinary specimens sent for gonococcal culture to the Department of Clinical Bacteriology, Central County Hospital, Örebro, Sweden. The strains were identified by oxidase testing, Gram stain, and immunofluorescent or fermentation procedures (18, 20; Danielsson, Ph.D. thesis). They were maintained on colonial morphology typing medium by the method of White and Kellogg (21) and on GC medium (BBL) (15). Slide agglutination tests with co-agglutinating reagents were performed on colonies from both of these media.

Other bacterial strains. Fifty oxidase-positive bacterial strains other than *N. gonorrhoeae* were also studied in the experiments. They represented the following bacterial species: 10 strains of *N. meningitidis* belonging to the serogroups A, B, C, and D; 15 strains of *N. pharyngis;* 7 strains of *Moraxella* species; 3 strains of *Haemophilus influenzae;* 3 strains of *Haemophilus parainfluenzae;* 12 strains of *Pseudomonas* species. The strains were maintained on ordinary blood agar and GC agar (BBL). Slide agglutination tests were performed with bacteria grown on GC agar plates. A meningococcal strain of serogroup B and a *Moraxella* strain (laboratory identification number 100101) were used for the absorption of anti-gonococcal sera and anti-gonococcal conjugates as described below.

Routine gonococcal cultures. Three thousand one hundred two rectal-genito-urinary and 173 tonsillo-pharyngeal specimens, sent for gonococcal culture analysis to the Department of Clinical Bacteriology, Central County Hospital, Örebro, Sweden, were grown on selective and nonselective gonococcal culture and media as described elsewhere (8). Routine identification of *N. gonorrhoeae* was made as follows. The GC agar plates were subjected to oxidase testing, and smears from oxidase-positive colonies were stained with fluorescein-labeled anti-gonococcal globulin (20; Danielsson, Ph.D. thesis). Genito-urinary specimens with oxidase-positive colonies and fluorescent antibody-positive smears showing gonococci with typical morphology were considered positive for *N. gonorrhoeae*. Tonsillo-pharyngeal specimens and rectal specimens with oxidase-positive and fluorescent antibody-positive smears were further subjected to fermentation procedures and other bacteriology tests (4, 18, 20, 22). All cultures showing oxidase-positive colonies were also subjected to slide agglutination tests with protein A-containing staphylococci coated with specific anti-gonococcal antibodies as described below. Unsensitized staphylococci or staphylococci coated with normal rabbit gamma globulin were used as control reagents.

Anti-gonococcal antisera. Anti-gonococcal antisera were obtained from rabbits immunized with formalin-treated, whole gonococcal organisms as described elsewhere (6). The sera were stored frozen at −62 C or −40 C. After thawing, the sera to be used for sensitization of reagent staphylococci were preserved by adding 1% (vol/vol) merthiolate (1:100) and then were kept at 4 C. Anti-gonococcal antiserum KÖ22 was selected for absorption experiments. Absorptions were carried out as follows. One volume of anti-gonococcal antiserum was mixed with 1 volume of *Moraxella* organisms and/or meningococcal group B organisms packed by centrifugation at 2,000 × *g* for 20 min. The mixture was incubated at 37 C for 4 h and overnight at 4 C (7). The absorbed serum was recovered by two centrifugations at 2,000 × *g* for 20 min.

IF procedures. The globulin portion of anti-gonococcal antiserum was precipitated by 45% saturation with $(NH_4)_2SO_4$ and then dissolved in distilled water. After dialysis against tap water and saline, the globulin was labeled by standard procedures using 0.0375 mg of fluorescein isothiocyanate (FITC) per mg of protein (16). A conjugate prepared from anti-gonococcal antiserum KÖ22 which was also used for the preparation of reagent staphylococci was selected for immunofluorescence (IF) tests. This conjugate had an F/P ratio of 14.7×10^{-3} (2). Absorption of the conjugated gamma globulin was carried out as described above for anti-gonococcal antiserum. Preparation and staining of smears followed standard procedures (6, 16). Fluorescence microscopy was performed with a Zeiss microscope equipped with a high-pressure mercury lamp (Osram HBO 200) and an oil dark-field condensor. A BG12 was used as a primary filter and a Zeiss 47 or Zeiss 50 was used as the secondary filter. The preparations were read at a magnification of ×420. Positive reactions were graded 1+ through 4+ as described before (6).

Preparation of co-agglutinating staphylococci. The stabilization and coating of protein A-containing staphylococci to make co-agglutinating reagent followed procedures described previously (14). The Cowan I strain of *Staphylococcus aureus* (NCTC 8530) was grown overnight in CCY broth (1), and the washed bacteria were treated for 3 h with 0.5% formaldehyde, washed in phosphate-buffered saline (PBS), pH 7.4, and exposed to 80 C for 4 min as described elsewhere (14). After additional washings the bacteria were suspended to 10% in PBS containing 0.1% sodium azide and kept at 4 C until use.

For coating of the protein A-containing staphylococci, 1 ml of the 10% suspension was added to 0.1 ml of unabsorbed or absorbed rabbit anti-gonococcal antiserum. After mixing the suspension, the staphylococci were washed twice and suspended to 1% in PBS containing 0.1% sodium azide. Control reagent consisted of uncoated staphylococci or staphylococci coated with gamma globulin from normal rabbit serum. The suspensions were stable for more than 6 months when stored at 4 C.

RESULTS

Co-agglutination reactions of oxidase-positive organisms with reagent staphylococci

coated with unabsorbed anti-gonococcal antibodies. One or more colonies of the oxidase-positive strains listed in Table 1, including 90 strains of *N. gonorrhoeae*, were emulsified in 2 drops of the 1% suspension of reagent staphylococci coated with unabsorbed anti-gonococcal antibodies. The slide was then tilted back and forth for approximately 30 s and observed with the naked eye. Controls were performed with reagent consisting of uncoated staphylococci or staphylococci coated with normal rabbit gamma globulin. The results obtained are summarized in Table 1.

All 90 gonococcal strains tested, regardless of colony morphology types, co-agglutinated with reagent staphylococci coated with unabsorbed anti-gonococcal antibodies (Table 1). In most instances a positive reaction occurred within 10 s and was fully developed within the next 20 to 30 s. Moderate (2+) to strong (3+) or very strong (4+) co-agglutinations were registered as well as some minor variations between various strains in the appearance of the agglutinates. No reactions occurred with uncoated staphylococci or with staphylococci coated with normal rabbit gamma globulin except on a few occasions where a weak auto-agglutination of a few gonococcal strains of colony morphology types 1 or 2 was observed. These reactions had a finely granular appearance not involving reagent staphylococci and was quite different from a true co-agglutination.

N. meningitidis strains of various serogroups gave weak (1+), moderate (2+), or strong (3+) co-agglutination reactions with reagent staphylococci coated with unabsorbed anti-gonococcal antibodies. A few apathogenic *Neisseria* strains spontaneously gave heavy aggregates when mixed with uncoated staphylococci or staphylococci coated with normal rabbit gamma globulin or anti-gonococcal antibodies. These aggregates did not involve reagent staphylococci and were easily distinguished by their character from true co-agglutination reactions. A few apathogenic *Neisseria* strains gave weakly positive (1+) co-agglutination reactions with the test reagent whereas some others remained homogeneous when mixed with control as well as test reagent.

Five strains of *Moraxella* species also formed heavy aggregates with both control and test reagent. The other two *Moraxella* strains included in the studies gave, with the test reagent, weakly to moderately positive reactions that were indistinguishable from a positive reaction with meningococci or gonococci. This was also true for some strains of *H. influenzae*. Negative, 1+, or 2+ reactions were noted with strains representing *H. parainfluenzae* and *Pseudomonas* species.

Comparison between results of co-agglutination tests with reagent staphylococci coated with absorbed anti-gonococcal antibodies and results of IF staining. The results

TABLE 1. *Coagglutination reactions of Neisseria gonorrhoeae and other oxidase-positive organisms with protein A-containing staphylococci, uncoated or coated with unabsorbed anti-gonococcal antibodies or normal rabbit gamma globulin*

Bacterial strains	No. of strains	Co-agglutination reactions with reagent staphylococci[a]				
		Uncoated	Coated with:			
			Normal rabbit gamma globulin	Unabsorbed anti-gonococcal antibodies		
				KÖ22	R85	R105
Neiserria gonorrhoeae	90					
Colony types T_1-T_2		−, OWA	−, OWA	+++, ++++	++, +++, ++++	++, +++, ++++
Colony types T_3-T_4		−	−	+++, ++++	++, +++, ++++	++, +++, ++++
Neisseria meningitidis (serogroups A, B, C, D)	10	−	−	+, ++, +++	+, ++, · ++	+, ++, +++
Neisseria pharyngis	11	−	−	−, occasional +	−, occasional +	−, occasional +
Neisseria pharyngis	4	SC	SC	SC	SC	SC
Moraxella species	5	SC	SC	SC	SC	SC
Moraxella species	1	−	−	++	++	++
Moraxella species	1	−	−	+	+	+
Haemophilus influenzae	3	−	−	+, ++	+, ++	+, ++
Haemophilus parainfluenzae	3	−	−	−, +	−, +	−, +
Pseudomonas species	12	−	−	−, +, ++	−, +, ++	−, +, ++

[a] Symbols: +, weak co-agglutination; ++, moderate co-agglutination; +++, strong co-agglutination; ++++, very strong co-agglutination. OWA, Occasional weak auto-agglutination; SC, spontaneous clumping.

obtained in co-agglutination tests with reagent staphylococci coated with anti-gonococcal antibodies, unabsorbed and absorbed with *Moraxella* and/or meningococcal organisms, are summarized in Table 2.

Staphylococci coated with anti-gonococcal antibodies absorbed with meningococci still gave co-agglutination reactions with gonococci but not with meningococci or apathogenic *Neisseria* strains. Co-agglutination occurred, however, with *Moraxella*, *H. influenzae*, and *Pseudomonas*. After absorption of the antiserum used for making reagent with both meningococcal and *Moraxella* organisms, co-agglutination was only obtained with gonococci (Table 2, Fig. 1). It should be noted, however, that the co-agglutination reactions developed more slowly with these absorbed reagent staphylococci. It usually took 20 to 30 s for the agglutination to appear whereas the same strains gave positive reactions within 5 to 10 s with the unabsorbed reagent. The reactions were also somewhat weaker with the absorbed reagent. Usually 2+ or 3+ reactions but occasional 1+ reactions were noted. By observing the slide for another 2 to 3 min the weak 1+ reactions developed, however, at least to 2+ reactions.

Fluorescent staining reactions obtained with FITC-labeled anti-gonococcal globulin from the same serum (KÖ22) used for coating of staphylococci are also summarized in Table 2. The unabsorbed conjugate gave 3+ to 4+ reactions with all gonococci tested. Reactions graded 2+ to 4+ were noted with meningococci. The *Moraxella* strain that gave a 2+ co-agglutination reaction with unabsorbed reagent staphylococci gave a 1+ to 3+ reaction with the anti-gonococcal conjugate. No reactions were observed with apathogenic *Neisseria* or *Pseudomonas* species, but a rather strong solid stain reaction with *H. influenzae* was noted.

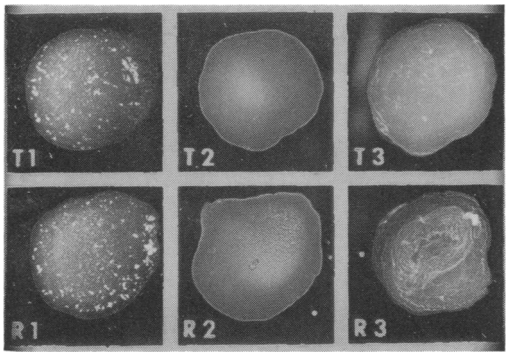

FIG. 1. *Co-agglutination patterns obtained with N. gonorrhoeae, N. meningitidis, and a Moraxella strain using protein A-containing staphylococci coated with anti-gonococcal antibodies, unabsorbed or absorbed with N. meningitidis and Moraxella organisms. Reagent staphylococci coated with unabsorbed antibodies show a 3+ reaction with N. gonorrhoeae (R1), a 1+ reaction with N. meningitidis (R2), and a 2+ reaction with a Moraxella strain (R3). Staphylococci coated with antibodies absorbed with N. meningitidis and Moraxella show a 3+ reaction with N. gonorrhoeae (T1), and negative reactions with N. meningitidis (T2) and Moraxella (T3).*

TABLE 2. *Results of testing Neisseria gonorrhoeae and other oxidase-positive organisms with co-agglutinating gonococcal reagent staphylococci and with FITC-labeled anti-gonococcus globulin*

Bacterial strains	No. of strains	Co-agglutination reactions of reagent staphylococci coated with anti-gonococcal antibodies			Staining reactions with FITC-labeled anti-gonococcal globulin	
		Not absorbed	Absorbed with:		Not absorbed (diluted 1:32)	Absorbed with *N. meningitidis* and *Moraxella* (diluted 1:8)
			N. meningitidis	*N. meningitidis* and *Moraxella*		
Neisseria gonorrhoeae (colony types T1, T2, T3, T4)	50	+++, ++++	++, +++	+, ++, +++	3 to 4+	3+
Neisseria meningitidis (serogroups A, B, C, D)	10	+, ++, +++	–	–	2 to 4+	–
Neisseria pharyngis	6	+	–	–	–	–
Moraxella species	1	++	++	–	1-3+	–
Moraxella species	1	+	+	–	–	–
Haemophilus influenzae	3	+, ++	+, ++	–	Solid stain	–
Pseudomonas species	4	+, ++	+, ++	–	–	–

After absorption of the conjugate with meningococci and *Moraxella* organisms, staining reactions were obtained only with gonococci. The IF titer of the conjugate dropped, however, two twofold dilution steps. The reactions obtained with the unabsorbed and absorbed conjugates corresponded well with co-agglutination results using unabsorbed and absorbed reagent staphylococci.

Identification of N. gonorrhoeae with specific reagent staphylococci in routine gonococcal cultures: comparison with IF staining and fermentation test. A total of 249 oxidase-positive colonies from 3,175 routine gonococcal cultures representing 3,102 specimens from the rectal-genito-urinary tract (urethra in males, and urethra, cervix, and rectum in females) and 173 tonsillo-pharyngeal specimens obtained from both males and females were tested in slide agglutination tests with reagent staphylococci coated with anti-gonococcal antibodies absorbed with meningococcal and *Moraxella* organisms. Control reagent consisted of uncoated staphylococci or staphylococci coated with normal rabbit gamma globulin. The results were compared with those obtained with IF tests or with Gram stain and fermentation procedures. The results are summarized in Table 3.

Reagent staphylococci coated with absorbed anti-gonococcal antibodies gave positive co-agglutination reactions only with *N. gonorrhoeae* and differentiated them from meningococci, apathogenic *Neisseria*, and other oxidase-positive bacteria as confirmed with IF tests or with Gram stain and fermentation procedures. This was true for gonococci isolated from the rectal-genito-urinary tract as well as from the tonsillo-pharyngeal region. A weak auto-agglutination of a few gonococcal strains occurred when the strains were emulsified in control reagent. It had the same character as the weak auto-agglutination observed with some gonococcal strains in experimental tests. It appeared as fine granules not involving reagent staphylococci and was quite different from the true co-agglutination, mostly grade 3+, shown by these strains. Examination of these colonies on colonial morphology typing medium indicated that they represented mainly type 2 colonies found to be rough in PBS or saline. A spontaneous formation of heavy aggregates of a different nature than auto-agglutination occurred with some apathogenic *Neisseria* strains and also with a few other oxidase-positive colonies representing gram-negative rods. This spontaneous clumping of some bacteria with uncoated as well as coated reagent staphylococci never interfered with the reading of the true co-agglutination reactions with gonococci.

DISCUSSION

A new serological technique based on the use of protein A-containing staphylococci coated with specific antibodies (14) has been adopted and utilized for the identification of *N. gonorrhoeae*, primarily detected as oxidase-positive colonies in gonococcal cultures. The use of

TABLE 3. *Comparison of results obtained with co-agglutination tests using specific gonococcal reagent staphylococci and with immunofluorescence or Gram stain and fermentation procedures in tests of oxidase-positive colonies from 3175 routine gonococcal cultures*

Source of specimen	No. of cultures with oxidase-positive colonies	Co-agglutination reactions of reagent staphylococci[a]			Diagnosis arrived at with immunofluorescence or Gram stain and fermentation
		Not coated	Coated with:		
			Normal rabbit gamma globulin	Anti-gonococcal antibodies absorbed with *N. meningitidis* and *Moraxella*	
Urethra, cervix, rectum	138	–	–	+, ++, +++	*N. gonorrhoeae*
	12	WA	WA	+++	*N. gonorrhoeae*
Pharynx, tonsils	9	–	–	+, ++, +++	*N. gonorrhoeae*
	21	–	–	–	*N. meningitidis*
Urethra, cervix, rectum	18	–	–	–	Organisms other
	6	SC	SC	SC	than *Neisseria*
Pharynx, tonsils	32	–	–	–	*N. pharyngis* or
	13	SC	SC	SC	gram-negative rods

[a] WA, Weak auto-agglutination; SC, spontaneous clumping.

reagent staphylococci coated with specific anti-gonococcal antibodies gave completely concordant results with Gram stain and fermentation procedures, and with specific FITC-labeled anti-gonococcal antibodies (Tables 2 and 3).

The described slide agglutination test constitutes a simple and rapid method for the laboratory culture diagnosis of *N. gonorrhoeae*. As compared to fermentation procedures it has the advantage of being more rapid and less laborious, and does not require complicated fermentation media. A positive diagnosis can be arrived at within a few minutes using specific gonococcal reagent staphylococci whereas 1 or more days are required for fermentation procedures.

The use of IF tests, basically also a serological technique, offers a morphological dimension and is nearly as rapid as the co-agglutination test. The latter one has the advantage of not requiring a fluorescence microscope. An IF test requires, however, less gonococcal material than a co-agglutination test. The IF method is therefore of advantage when only one or a few oxidase-positive colonies are available. On the other hand, in rectal and tonsillo-pharyngeal gonorrhoea, two well-documented clinical entities (4, 13, 22), IF tests cannot be used alone but must, when positive, be supplemented with fermentation tests and perhaps other bacteriological examinations, since in many of these specimens gonococci have to be differentiated from meningococci, apathogenic *Neisseria*, and some other oxidase-positive organisms (4, 18, 20, 22). The results of the present investigation show that co-agglutination tests are of great value on these occasions.

It is well known that colony morphology types T1 and T2 of *N. gonorrhoeae* become auto-agglutinated when grown in liquid media or when suspended in saline or PBS, a reaction which seems to be mediated by zones of adhesion on the gonococcal cell walls and possibly accentuated by the presence of pili (12, 19). The auto-agglutination in saline or PBS was also demonstrated with T1 and T2 gonococcal colonies used in this study, although most of these strains were homogeneous when they were mixed with a suspension of nonreacting staphylococci. On a few occasions a slight auto-agglutination was observed with T2 colonies which, however, contrasted to the true and usually strong co-agglutination of these strains with specific reagent. The advantage of the co-agglutination method for analyses of auto-agglutinating strains was pointed out by Christensen et al. (5) and by Juhlin and Winblad (11).

A spontaneous clumping with specific reagent staphylococci as well as with uncoated and control staphylococci occurred with some apathogenic *Neisseria*, some *Moraxella* strains, and also some other oxidase-positive bacteria. The formation of such aggregates, some of which were rather heavy, also contrasted in character to the true co-agglutination of gonococci with specific reagent staphylococci.

The results of our experiments show that reagent staphylococci coated with unabsorbed anti-gonococcal antibodies gave moderate to strong reactions with *N. meningitidis* and also with some *Moraxella*, *Haemophilus*, and *Pseudomonas* strains. Cross-reactions with meningococci were expected since it is well known that gonococci and meningococci share many antigens (6, 7, 20, 23). Absorption of the antiserum with meningococci eliminated the cross-reactions with these strains but not those with *Moraxella*, *Haemophilus*, or *Pseudomonas* organisms until anti-gonococcal antibodies were also absorbed with *Moraxella* organisms. Further investigation has shown that the same results are arrived at by absorption with *H. influenzae* or *Pseudomonas* organisms instead of *Moraxella*. The observed serological cross-reactions with these organisms and the elimination of these reactions by absorption indicate an antigenic relationship between these bacteria and gonococci. These findings were supported by results using IF tests and will be subjected to further study.

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