Radioimmunoassay for Detection of Antibodies to *Neisseria gonorrhoeae*

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A radioimmunoassay has been developed and evaluated for the serological diagnosis of gonorrhea. Purified gonococcal antigen was obtained from a culture of *Neisseria gonorrhoeae* (B370) and labeled with ^{125}I for use in a double-antibody test system. The test was evaluated in populations segregated by sex and risk. The specificity of the assay in females was 90.2% (55/61) in low risk, 82.2% (2,245/2,732) in medium risk, and 54.1% (335/619) in high risk. The sensitivity was 69% (20/29) in medium risk and 78.3% (288/367) in high risk. In males, test specificity was 92.3% (24/26) in low risk and 50% (48/96) in high risk. The sensitivity was 70.8% (143/202) in the high-risk group. The data in this study indicate that this assay should not be employed for screening of either high- or medium-risk populations.

Of the infectious diseases confronting the human race today, gonorrhea represents one of the major public health problems. This problem is not restricted to the United States alone, but is worldwide. Current incidence figures indicate that it is the highest in 53 years. Thirty percent of these cases constitute reinfection. Although there are over a half million cases reported in the United States, it is estimated that another two million go unreported (9).

Until recently, little effort was placed on developing improved methods of detection, diagnosis, or prevention. The current method of detection includes Gram stain of urethral exudates, culture of vaginal specimens, and interpretation of symptoms (5). The sensitivity of smear and culture techniques can be highly variable, depending on where and by whom the specimens are collected and processed. They can range from as low as 40 to 90% (22, 25). Diagnosis by symptomology and case history is probably the easiest; however, the public health problem does not lie with the apparent cases of gonorrhea but with asymptomatic individuals. Additional sources of infection often go undetected because appropriate culture sites are not included, such as the pharynx and rectum (13, 17, 27).

The detection problem is complicated by the lack of an easy, reliable method of screening the population for gonorrhea. Since 10 to 20% of infected males are asymptomatic (6, 14) and as many as 40% of infected females are also asymptomatic (3, 5, 26), there is an urgent need for a rapid screening test. Currently, the only methods for detecting this reservoir of asymptomatic

individuals is by the costly use of smear and culture and the location of contacts.

The present report describes the development and evaluation of a radioimmunoassay (RIA) to detect antibodies to gonorrhea in symptomatic and asymptomatic males and females. The procedure employs the gonococcal (GC) antigen described by Chen et al. (4).

MATERIALS AND METHODS

Preparation of charcoal medium. To 1,000 ml of distilled water, 36 g of GC agar base (Difco Laboratories), 5 g of agar (Difco), and 5 g of activated charcoal were added. The agar was heated to boiling to dissolve the ingredients and autoclaved at 121°C for 15 min. It was allowed to cool to 50°C, and 10 ml of IsoVitaleX was added.

Growth of *Neisseria gonorrhoeae*. Cultures of *N. gonorrhoeae* B370 (ATCC 21824) were streaked on charcoal plates and incubated at 35°C in a CO₂ incubator for 48 h. They were subcultured to charcoal agar slants and incubated at 35°C in a CO₂ incubator for 18 to 24 h. The cells were then subcultured into a 75-cm² tissue culture flask containing 50 ml of charcoal agar. After incubation, 7 ml of sterile saline was added to the tissue culture flask, and the cell suspension was removed, inoculated onto a stainless steel tray (24 by 24 in. [ca. 60.96 cm]) containing 1 liter of charcoal medium and incubated for 18 h at 35°C in a CO₂ incubator.

Harvesting the cells. The cells were harvested with saline, and a Gram stain was performed to assure purity. Antigenicity was monitored by indirect immunofluorescence by the method of Gaafar and D'Arcangelis (11).

Extraction of antigen. The cell suspension was filtered through two plies of gauze and was centrifuged at 4,100 × g in a Sorvall RC 2-B centrifuge at 5°C for

10 min. The cell pellet was suspended in 2 ml of saline per g (wet weight) of cells. An equal volume of 0.6% (wt/vol) sodium dodecyl sulfate (SDS) in saline was added to the cell suspension and stirred at 25°C for 10 min. The suspension was then centrifuged at $4,100 \times g$ at 5°C for 10 min. The supernatant fluid was decanted and refrigerated. The cells were reextracted with a volume of 0.1% SDS equal to the original volume of 0.6% SDS, stirred for an additional 10 min, and centrifuged as before. The supernatant fluids were pooled and centrifuged at $12,100 \times g$ for 20 min at 5°C. Sufficient 2% sodium azide was added to the supernatant fluid to give a final concentration of 0.02%. The extract was concentrated to 1 to 1.5 mg of protein per ml by Ficoll dialysis. Protein determination was by the Lowry protein assay (19).

Antigen purification. The GC antigen was purified by a modification of the procedure of Chen et al. (4). Void volume fractions from Sepharose 4B were pooled and digested with DNase and RNase. The partially purified GC antigen was mixed with 0.5 mg of DNase and 0.25 mg of RNase and dialyzed overnight at room temperature against 0.05 M NH_4HCO_3 containing 0.01 M MgCl_2 and 0.1% NaN_3 . The nuclease-treated antigen was then separated on a column (2 by 42 cm) of Bio-Gel A-1.5 M (100 to 200 mesh; Bio-Rad Laboratories). The column was eluted at 4°C with 0.05 M NH_4HCO_3 at a flow rate of 10 ml/h. Void volume fractions were pooled, concentrated by Amicon ultrafiltration (PM10 filter), and stored at -20°C. Elution profiles from the gel filtration indicated that the molecular weight of the GC antigen was in excess of 5×10^6 .

Iodination. Purified GC antigen was iodinated by a modification of the Hunter and Greenwood procedure (16). To 100 μl of 0.5 M phosphate buffer (pH 7.5) were added 10 μg of antigen, 0.5 mCi of Na^{125}I (Amersham Corp.), and 10 μl of chloramine-T (500 $\mu\text{g}/\text{ml}$). After 1 min of incubation at room temperature, the reaction was terminated by the addition of 250 μl of sodium metabisulfite (100 $\mu\text{g}/\text{ml}$). After an additional 30 s of incubation, 100 μl of K1 (1 mg/ml) and 20 μl of 10% bovine serum albumin (BSA) solution (Pentex; fraction V) were added to the reaction mixture. The iodinated antigen was separated on a column (1 by 30 cm) of Bio-Gel A-1.5 M (Bio-Rad; 100 to 200 mesh). Elution was done with 0.05 M phosphate buffer (pH 7.0) at a flow rate of 20 ml/h. Fractions containing the labeled GC antigen were pooled and diluted with a 2% BSA solution in 0.01 M EDTA with 0.1% NaN_3 . The ^{125}I -GC antigen was routinely stored at -20°C because storage at either 4°C or room temperature resulted in its deterioration. This instability, which became apparent within several days, was characterized by a profound loss of binding to GC-positive sera and an increase in nonspecific binding to GC-negative sera. Before use, the antigen was diluted in the BSA solution to approximately 11,400 dpm/50 μl .

RIA procedure. Five microliters of serum sample was added to 100 μl of 0.01 M phosphate-buffered saline, pH 7.2. Ten microliters of goat anti-human immunoglobulin G (IgG) serum diluted in normal goat serum (Granite Biologicals) was added. To this solution, 50 μl of ^{125}I -GC label was added, gently mixed, and incubated overnight at 4°C. At the end of the incubation period, 500 μl of phosphate-buffered saline was added to facilitate sample handling, and the solution was filtered. Whatman GF/C filter paper (2.4 cm)

was placed on a membrane filter manifold (Millipore Corp.) and soaked for 5 min with a 2% BSA solution in 0.01 M EDTA with 0.1% NaN_3 . The BSA was removed by the application of vacuum (150 mm Hg [ca. 8.0 kPa]), and the sample was applied to the filter. The filter was then washed with deionized water, dried slightly under vacuum, and counted in a gamma scintillation counter (Searle, model 1285).

Second antibody titration. The concentration of second antibody used in the RIA was determined by testing each newly iodinated batch of partially purified antigen with goat anti-human IgG diluted in normal goat serum in the range of 1:2 to 1:128. The optimal dilution was that which maximized the positive sera binding while minimizing nonspecific reactivity.

Study population. Sera for this study were collected by venipuncture from participants who had given their consent to participate in this study. At the same time, cervical or urethral cultures or both and smears were performed. The participants were seen by physicians in family planning clinics, obstetrics-gynecology clinics, venereal disease clinics, student health services, and private practice. Sera for the low-risk population were obtained from celibates from religious cloisters.

A low-risk population is comprised of men or women who are living within the confines of a cloister, children between the ages of 2 and 12, or a population in which the frequency of gonorrhea is 0.8% or lower. A moderate-risk population contains individuals obtaining a routine physical or a population of individuals in which the frequency of a positive smear or culture or both is less than 1.5%. A high-risk population is defined as one in which the individuals having past exposure are attending a venereal disease clinic or a population in which the frequency is 20% or higher. The overall rate of gonorrhea in the general population is about 4.7% (30). A case of gonorrhea was defined as a positive culture or smear or both.

Calculations and data analysis. The patients were divided into four basic categories: patients whose samples gave a positive RIA and who had gonorrhea (A); patients whose samples had a negative RIA and who had gonorrhea (B); those whose samples gave a positive RIA and who did not have gonorrhea (C); and those whose samples gave a negative RIA and who did not have gonorrhea (D). The calculations were made as follows: % prevalence = $(A + B)/(A + B + C + D) \times 100$; % positive = $(A + C)/(A + B + C + D) \times 100$; % false negative = $B/(A + B) \times 100$; % correctly diagnosed = $(A + D)/(A + B + C + D) \times 100$; sensitivity = $A/(A + B) \times 100$; specificity = $D/(C + D) \times 100$; predictive value = $A/(A + C) \times 100$.

Cross-reactivity studies. *N. meningitidis*, groups A, B, C, and D, were grown on charcoal media and extracted with SDS by the procedure of Chen et al. (4). Extracts were then tested for their ability to inhibit the binding of a strongly positive serum with labeled GC antigen in the RIA procedure.

Absorption of antibody. The ability of *N. gonorrhoeae* to remove the antibodies present in 10 sera from culture-positive patients was assessed. *N. gonorrhoeae* B370 was grown on charcoal media and harvested with 2% Formalin fixative. A 1-ml amount of each of 10 culture-positive sera was absorbed with 0.1 ml of packed cells by incubating for 30 min at 37°C with shaking. The sera were then centrifuged, the supernatant fluids were withdrawn, and the above

procedure was repeated two more times. The supernatants were then assayed for antibody content in the RIA.

RESULTS

Yields. The yield of cells per liter of medium was between 1.5 and 3.0 g. This in turn yielded between 26 and 34 mg of crude protein. Starting with approximately 300 mg of crude antigen, 20 to 40 mg of purified antigen was obtained. For every 10 μ g of purified antigen iodinated, sufficient labeled antigen was produced to perform between 8,000 and 10,000 tests.

Second antibody titration. In the course of titrating the goat anti-human IgG serum, it was observed that maximum binding of the GC label by human GC antibodies was not obtained at the equivalence point for the precipitation of human IgG-goat anti-IgG complexes. As seen in Fig. 1, maximum binding of the label occurred when 0.16 μ l of goat anti-human IgG was added. No visible precipitate could be observed at this point. Optimum precipitation of human IgG-goat anti-human IgG complexes did not occur until 40 μ l of goat anti-human IgG had been added. The dose-response relationship for the binding of the GC label to antibody was observed between 0.04 and 0.63 μ l of second antibody. In the above volume range, it appears that large soluble complexes of 125 I-GC, human anti-GC, and goat anti-human IgG formed during the incubation, and these were retained by the filter during the separation step. The formation of these complexes can be referred to as "nonvisible aggluti-

nation" since the antigen molecule is very large (molecular weight, 5,000,000) as compared to the antibody molecule.

Negative sera (from individuals with negative GC cultures and no previous history of the disease) showed a slight reaction with the 125 I-GC antigen, indicating the presence of cross-reacting antibodies (Fig. 2). To avoid interference by these nonspecific antibodies, the volume of goat anti-human IgG serum was chosen so as to minimize the formation of nonvisible agglutinates with negative sera.

A series of sera of culture-positive and -negative patients were compared, using the optimal volume of goat anti-human IgG serum necessary for the formation of 125 I-GC antigen containing either agglutinates or precipitates (Table 1). As expected, the binding obtained for the culture-positive samples was significantly higher when using the optimal volume necessary for nonvisible agglutination as opposed to precipitation.

Cross-reactivity studies. Crude SDS outer membrane extracts of the four most common serogroups of *N. meningitidis* (groups A, B, C, and D) were tested for their ability to inhibit the binding of 125 I-GC antigen to the specific antibodies in a positive serum sample. The results of testing undilute crude extracts and serial dilutions are shown in Table 2. Groups B and C were highly inhibitory, indicating the presence of cross-reacting antigenic determinants. Groups A and D also inhibited, but to a lesser degree. No attempts were made to identify or characterize

FIG. 1. Comparison of nonvisible agglutination and precipitation reactions. A culture-positive serum (5 μ l) was incubated overnight at 4°C with various amounts of goat anti-human IgG and 50 μ l of 125 I-GC antigen (8,000 cpm). The amount of radioactivity in the filters (●) is compared with the protein concentration of precipitates (○).

FIG. 2. Titration curves of goat anti-human IgG serum. Culture-positive (○) and -negative (●) sera (5 μ l each) were incubated overnight at 4°C with various amounts of goat anti-human IgG serum and 50 μ l of 125 I-GC antigen (8,000 cpm). The arrow corresponds to the volume of second antibody normally used to avoid interference by cross-reacting antibodies present in culture-negative sera.

these cross-reacting antigens within the serogroups tested.

Absorption experiment. The results of absorbing 10 culture-positive sera with *N. gonorrhoeae* cells are presented in Table 3. Without exception, absorption with the bacteria resulted in the removal of antibodies capable of binding to the 125 I-GC antigen.

Precision. The reproducibility of the assay was evaluated by analyzing a pool of culture-positive

sera. The pool was analyzed 10 times on a given day for intraassay precision and daily over a week for interassay precision. The coefficients

TABLE 2. Cross-reactivity study with *N. meningitidis* groups A, B, C, and D

Sample	Filter cpm	% Inhibition
Positive control	2,455	
<i>N. meningitidis</i> group A		
Undiluted	754	69.3
1:10	951	61.3
1:100	2,050	16.5
1:1,000	2,060	16.1
1:10,000	1,724	29.8
<i>N. meningitidis</i> group B		
Undiluted	364	85.2
1:10	1,133	53.8
1:100	1,176	52.1
1:1,000	1,472	40.0
1:10,000	1,623	33.9
<i>N. meningitidis</i> group C		
Undiluted	187	92.4
1:10	983	60.0
1:100	1,207	50.8
1:1,000	1,612	34.3
1:10,000	2,399	2.3
<i>N. meningitidis</i> group D		
Undiluted	1,308	46.7
1:10	1,463	40.4
1:100	2,032	17.2
1:1,000	2,305	6.1
1:10,000	2,475	0

TABLE 1. Comparison of RIA results using optimum levels of goat anti-human IgG for nonvisible agglutination and precipitation

Sample	Culture	Nonvisible agglutination filter cpm	Precipitation filter cpm
1	—	123	165
2	—	98	137
3	—	120	129
4	—	204	97
5	—	207	154
6	+	1,000	204
7	+	524	97
8	+	788	225
9	+	817	111
10	+	662	575
11	+	915	444
12	+	789	534
13	+	2,319	1,783
14	+	2,266	698
15	+	2,206	394
16	+	2,052	571

TABLE 3. Absorption of culture-positive sera with *N. gonorrhoeae*

Sample	Filter cpm before absorption	Filter cpm after absorption
1	2,096	283 ^a
2	4,909	278
3	4,441	297
4	1,927	286
5	3,259	173
6	4,501	307
7	4,738	262
8	5,985	235
9	2,599	229
10	5,215	210

^a Sera from 10 culture-positive individuals were absorbed with 0.1 ml of packed *N. gonorrhoeae* cells as described in the text. Filters counts per minute between 200 and 300 are representative of background counts.

of variation for intra- and interassay precision were 6.8 and 15.5%, respectively.

Selecting a cutoff. For estimation of sensitivity and specificity, a cutoff was chosen from the distribution of counts per minute results from sera of culture-positive and culture-negative patients.

An approach that is commonly cited is to select a cutoff value that will minimize the average cost of misclassifying a randomly

screened patient (21). When this approach was applied to our study, the cutoff chosen was 400 cpm. Sera giving results between 400 and 600 cpm were considered borderline but classified as positive. The cutoff was applicable for all runs since: (i) the labeled antigen was only used if the time from labeling was less than 4 weeks, and (ii) reference reagents were included with each run.

Test performance. The data presented in Fig. 3 and Table 4 show the overall performance of the assay in the general population, in all males and females. In all patients assayed ($n = 4,135$), the prevalence of disease was 14.5%, with 599 individuals positive for gonorrhea by culture or smear. The overall assay sensitivity was 75.3% (451/599) with a specificity of 76.6% (2,709/3,536). Sensitivity of the assay in males was 70.8% (143/202). Since a large proportion of males assayed were from a high-risk population, the prevalence of disease was 62.0% (202/326). Due to this large proportion of high-risk males, many with prior history of disease, the assay specificity was 59.7% (74/124), indicative of the persistence of antibody from previous exposure. In the overall female population, the prevalence of disease was 10.4% (397/3,809). The assay sensitivity was 77.6% (308/397) with a specificity of 77.2% (2,635/3,412).

The performance of the assay on males, segregated by degree of risk, is shown in Table 4.

FIG. 3. Histogram comparing the distribution of counts per minute results for culture-positive and culture-negative cases in all populations studied.

TABLE 4. Performance of test by sex and degree of risk

Patient population	Total no. of patients	No. with disease	% Prevalence	No. positive by RIA	% Sensitivity	No. negative by RIA	% False negative	No. without disease	No. negative by RIA	% Specificity	No. positive by RIA	% False positives	No. correctly identified	% Correctly identified
Overall population	4,135	599	14.5	451	75.3	148	24.7	3,536	2,709	76.6	827	23.4	3,160	76.4
Males, all	326	202	62	143	70.8	59	29.2	124	74	59.7	50	40.3	217	66.6
Low risk	26	0	— ^a	—	—	—	—	26	24	92.3	2	7.7	24	92.3
Medium risk	2	0	—	—	—	—	—	2	2	100	0	0	2	100
High risk	298	202	67.8	143	70.8	59	29.2	96	48	50	48	50	191	64.1
Females, all	3,809	397	10.4	308	77.6	89	22.4	3,412	2,635	77.2	777	22.8	2,943	77.3
Low risk	61	0	—	—	—	—	—	61	55	90.16	6	9.84	55	90.16
Medium risk	2,761	29	1.05	20	69	9	31	2,732	2,245	82.2	487	17.8	2,265	82
High risk	987	368	37.3	288	78.3	80	21.7	619	335	54.1	284	45.9	623	63.1

^a —, Not done.

Although the number of samples from a low-risk population was small, the specificity of the assay in this population was quite high, 92.3% (24/28). In the high-risk population, the specificity was 50.0% (48/96), and the sensitivity was 70.8% (143/202). There was no disease present in low- or medium-risk males.

The performance of the assay in females, segregated by risk, is also shown in Table 4. There were no cases of gonorrhea reported for the low-risk population, and the prevalence of disease in medium- and high-risk populations was 1.1% (29/276) and 37.3% (368/987), respectively. The specificity of the assay in the low-risk population was 95.1% (55/61). In the medium-risk population the specificity was 82.2% (2,245/2,732), and the sensitivity was 69.0% (20/29). The specificity decreased to 54.1% (335/619), whereas the sensitivity increased to 78.3% (288/360) in the high-risk population.

When smear results were compared with culture results, culture failed to pick up a positive case of gonorrhea 5.5% (25/455) of the time. The RIA identified as positive 80% (20/25) of those cases missed by culture. If both culture and RIA are combined, the probability of missing a case is substantially reduced.

DISCUSSION

The RIA procedure is sensitive, reproducible, and easy to perform. The manifold filtration used at the end of the incubation period to separate bound from free GC label was chosen for two reasons. First, it allows for the simultaneous processing of multiple samples at a much faster rate than centrifugation wash cycles. Secondly, it avoided the problem of co-precipitation of unbound GC label during centrifugation, due mainly to its large molecular weight.

When titrating goat anti-human IgG serum to determine the optimum volume to be used in the RIA, it was observed that maximum binding of the GC label by human GC antibodies was not obtained at the equivalence point for the precipitation of human IgG-goat anti-IgG complexes. A plausible explanation for this is the formation of nonvisible aggregates of GC label-human anti-GC-goat anti-human IgG. As expected, these aggregates would require an optimum ratio of goat anti-human antibodies to human GC antibodies to react with the maximum number of GC label molecules. Therefore, the size of the aggregates would become smaller as one moves into second antibody excess. Separate from this nonvisible-agglutination phenomenon involving the GC antigen, visible precipitates result from the bulk of the human gamma globulins combining with second antibody molecules to form large lattices, which subsequently precipitate.

The antibodies being detected and quantitated in the RIA are directed against *N. gonorrhoeae* since absorption of sera from culture-positive patients by cells of *N. gonorrhoeae* completely removes the reactivity with labeled GC antigen. Existence of antigenic determinants shared with other *Neisseria* species was shown in experiments with *N. meningitidis* extracts, which strongly inhibited the binding of GC label by sera from culture-positive patients. The presence of antibodies to these common determinants could easily explain the false-positive reactions observed with some sera from culture-negative patients.

In this study, the ability of the assay to detect the presence of antibodies to *N. gonorrhoeae* in human serum as an indication of possible infection was assessed in over 4,000 patients. The performance of the test turned out to be primarily a function of the prevalence of the disease in the population being tested. For example, in a low-risk female population, the percent correctly diagnosed was 90, whereas in a medium-risk population it was only 82, and in a high-risk population it dropped to 50. The predictive value, on the other hand, was only 3.9% in the medium-risk group, whereas it was as high as 50% in the high-risk group. The high incidence of positive test results due to residual antibody from previous infections is one of the major contributing factors to the unacceptable test performance, especially in high-risk populations where approximately 49% of the patients have had repeated infections with *N. gonorrhoeae*. Since this test measures antibodies to gonococci, it cannot differentiate between present and past infections.

It is difficult to compare results of other serological tests for gonorrhea due to differences in the populations studied. Using an indirect fluorescence test, Gaafar (10) reported a sensitivity of 95% for women and 86.9% for men in a selected population of culture-positive individuals. This represents a population with a prevalence of 100%. Similar results for an RIA were reported by Luoma et al. (20), again in a preselected population of culture-positive and -negative sera.

The prevalence of the disease in the entire population examined in our study was 14.5%, and the sensitivity of the assay was 75.3%, which is similar to that obtained with fluorescence tests by other investigators who did not preselect their study populations (2, 15, 29). A comprehensive review of the sensitivity and specificity of various serological tests for gonorrhea has been prepared by Koransky et al. (18).

Culture is recognized as a satisfactory procedure for the diagnosis of gonorrhea in females, but it is impractical in terms of time, cost, and

efficiency. It is not routinely used in the general female population, thus making it impossible to detect the targeted population of asymptomatic females (22). A recent report (6) has stressed the need for a more aggressive search for asymptomatic carriers.

Outside of specialized clinics, culture may be undependable. Reports have shown that there is a 1.6 to 6.4% decrease in recovery rate of organisms, due to a delay in incubation, when cultures must be transported to a laboratory for processing (7). Among the other factors limiting the ability to recover *N. gonorrhoeae* are the concentration of ambient carbon dioxide and the type of media (8, 28). Nine percent of the strains isolated are sensitive to hemoglobin (23), 10% are sensitive to vancomycin (1, 7, 24), and 10% have specific nutritional requirements not met by conventional media (6, 12).

The present assay was developed to detect antibodies to *N. gonorrhoeae* in large populations of individuals who would not be routinely cultured for gonorrhea. Unfortunately, the data obtained in this comprehensive study indicate that the assay should not be employed for screening purposes in either high- or medium-risk populations. The test has a low specificity in the high-risk group, resulting from the presence of residual antibody in serum from previous infections. Although in the medium-risk group the percent correctly diagnosed is as high as 82%, the low predictive value of 3.9% makes the test performance unacceptable. Culture remains then as the only presently available tool for the diagnosis of infection with *N. gonorrhoeae*.

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