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Laboratory Diagnosis of Gonococcal Infection by Genetic Transformation

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The transformation test for the detection of infection by *Neisseria gonorrhoeae* has been examined using *pro* gonococci as recipients and DNA preparations from 912 clinical isolates and from 240 direct swab specimens as donors. The reliability of the method was checked with DNA from clinical isolates; 82% of the *N. meningitidis* from throat swab specimens were capable of transforming the gonococcal recipients, but after identification of the meningococcus by the aminopeptidase profile, the transformation test was then 99.5% positive for the gonococcus with virtually no false-positives. The only other organism to give a positive reaction was *N. lactamica*, which occurred once in 912 specimens. When applied directly to swab specimens, the reliability of the test was reduced, but this may have been related to variability of the specimen itself. However, 7 of 15 specimens which were microscopically suspected to be gonococci but unculturable were positive; also, 9 out of 38 unculturable specimens that were not even suspected to be gonococci were positive. Hence the test was able to identify the presence of gonococci that were unculturable. The aminopeptidase activities were not sensitive enough to be detected in the direct swab specimens, and neither *cys* nor *leu* auxotrophs were suitable as recipients to give a differentiation between *N. gonorrhoeae* and *N. meningitidis*. Evidence was obtained which would support the proposition that the transfer of genetic material between *N. gonorrhoeae* and *N. meningitidis* may occur.

There are still many cases of suspected gonococcal infection in which a positive diagnosis cannot be made because the organism cannot be cultured from the clinical specimen. The reports by Janik et al. (6) and Bawdon et al. (1) indicated that a technique based on the genetic transformation of an auxotrophic *Neisseria gonorrhoeae* as recipient to crude preparations of DNA from clinical specimens may prove useful. It was shown that transformation could also be effected by the DNA prepared directly from the specimen on the swab, so that the need for viable specimens would be eliminated. Hence, it was of interest to submit the transformation method to a survey of clinical specimens and assess its suitability as a routine laboratory procedure.

MATERIALS AND METHODS

Donor material. Clinical specimens were of two types: (i) isolates from St. Mary's Hospital Medical School, London (which were derived from swabs from the venereal disease clinic at St. Mary's Hospital) and from the Middlesex Hospital, London. All specimens had been identified by the isolating laboratory using cultural, immunofluorescence, and sugar tests, and, in some cases, the aminopeptidase profile; (ii) patient's swabs directly immersed in 0.025% sodium dodecyl sulfate in 0.15 M NaCl and 0.015 M trisodium citrate (the SDS solution), from the venereal disease clinics at the Middlesex Hospital and the Westminster Hospital in London, and the Dreadnought Seamens' Hospital, Greenwich. All swabs were "Hospiswabs" (Medical Wire and Equipment Co. Ltd., Corsham, Wilts) and were supplied to all the clinics by this laboratory. The patient's diagnosis was made by the respective hospital laboratories from other swabs taken at the same time.

Cultures were grown mainly on GC medium base (Difco) with Kelloggs defined supplements 1 and 2, but sometimes Columbia medium (Oxoid) with 5% (vol/vol) defibrinated horse blood, and incubated at 37°C in an atmosphere containing 7% CO_2 and a high humidity. The crude donor DNA preparations were prepared from either (i) isolated colonies made into a paste in SDS solution and heated at 60°C for 1 h in a water bath, or (ii) the swab samples immersed directly in the SDS solution and heated in the same way.

Recipient organisms. (i) Two proline-requiring *N. gonorrhoeae* (GC) designated 9184 and 9248 were isolated from the clinical isolates; they cross-transformed each other to pro^+ at high frequency and were both used for testing each clinical specimen, thus eliminating a possible negative transformation test due to the patient's specimen possessing an identical auxotrophic genotype. (ii) One leucine-requiring GC, designated 1059 *leu*, was obtained as a spontaneous mutant from the clinical isolate 1059 wild type for leucine, proline, arginine, uracil, and thiamine pyrophosphate.

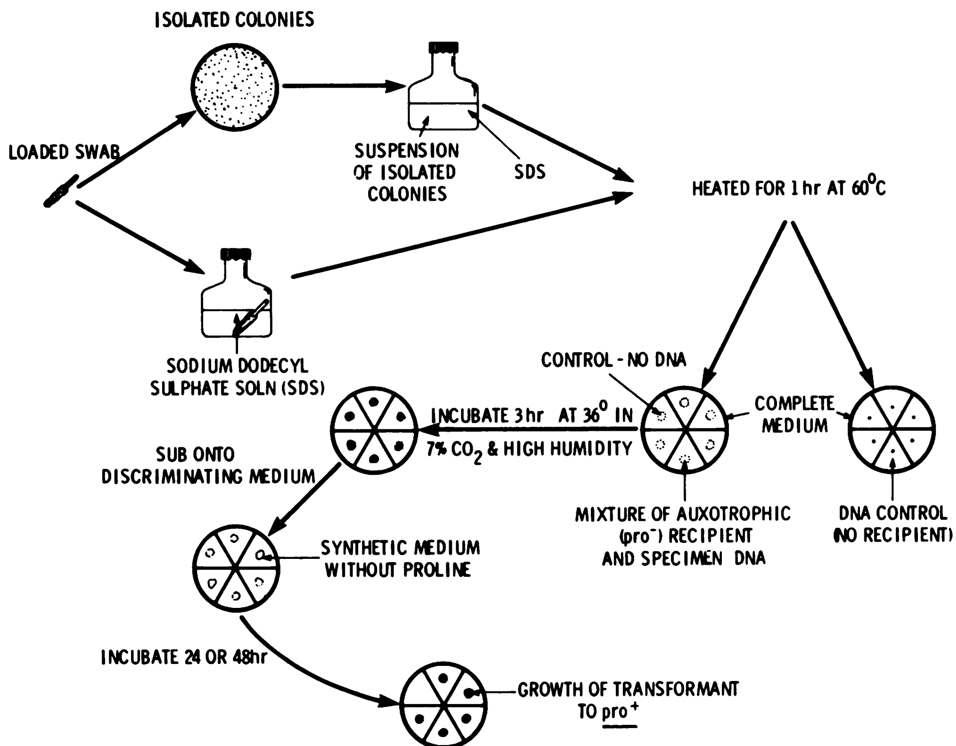

FIG. 1. Diagram of transformation assay.

Synthetic media. The medium of La Scolea and Young (7) was first used, but different batches gave such varying levels of growth when inoculated with the same GC isolates that it was considered too inconsistent for this purpose. The complex medium of Catlin (2) was then modified to give a slightly less complex medium which gave consistent growth characteristics with the same set of GC isolates. It was found that sodium lactate, glycerol, polyvinyl alcohol, and Tween 80 (i.e., solution 3 of Catlin) could be omitted, and also hemin and 2,2',2"-nitrilotriethanol (i.e., most of solution 6 of Catlin).

Transformation assay. The method used for transformation assay is diagrammatically represented in Fig. 1 and closely followed that described by Janik et al. (6). A petri dish containing complete medium was divided into segments, and each segment was inoculated with a loopful of a suspension of the recipient *pro* organism, a different plate being used for each recipient. To each sector was then added a clinically derived donor DNA preparation and mixed in, one sector being left with no DNA as the control. A second control plate was made to which the DNA preparations were added but no recipient organism. After 3 h of incubation at 36°C in 7% CO_2 in humid conditions, a smear from each segment was inoculated onto plates containing synthetic medium lacking proline. After 24 and 48 h of incubation in 7% CO_2, growth of any pro^+ organism was noted.

Assay of aminopeptidase activity. The aminopeptidase activities of suspensions of the clinical isolates were assayed, using γ-L-glutamyl-4-nitroanilide, L-arginine-β-naphthylamide HCl, and L-4-hydroxyprolyl-β-naphthylamide as substrates, by a modification of the method of D'Amato et al. (4) (Cathy Ison, personal communication). A 50-μl volume of the bacterial suspensions (in 0.85% [wt/vol] saline) was mixed with 50 μl of each of the substrates, and, after incubating at 36°C for 2 to 4 h under humid conditions, 25 μl of 0.4 N HCl and 25 μl of 0.08% (wt/vol) $NaNO_2$ were added and placed at 4°C for 10 min. On the addition of 50 μl of 2% (wt/vol) ammonium sulfamate and 25 μl of 1.6% (wt/vol) naphthylene diamine dihydrochloride in methanol, the development of the appropriate color for each substrate showed a positive reaction. All *Neisseria* possess the L-arginine aminopeptidase, but the gonococcus does not show the L-glutamylaminopeptidase activity possessed by the meningococcus, but splits the L-4-hydroxyprolyl-β-naphthylamide.

RESULTS

Transformation using clinical isolates. A preliminary survey was made of 626 specimens from St. Mary's Hospital and Middlesex Hospital, London, taken from the cervix, urethra (male and female), and throat, the organisms being identified by the isolating laboratory. After ascertaining the best conditions for the transformation procedures with known gonococcal specimens, the vast majority of them were then handed to us "blind." The composition of this group of specimens was made up as follows:

TABLE 1. Clinical isolates giving positive or negative transformation of the *pro* gonococcus recipients to pro^+

Isolate	No. of specimens[a]	% Total	% Each species
GC[b]			
Transformation positive	459	78.2	91.1
Transformation negative[c]	45	7.7	8.9
MG (false transformation positive)	82	14.0	
Others (false transformation positive)	1[d]	0.2	

[a] Total tested = 587.
[b] Identified by cultural, immunofluorescence, sugar, and aminopeptidase reactions.
[c] False-negatives.
[d] *N. lactamica.*

81% *N. gonorrhoeae* (GC), 18% *N. meningitidis* (MG), and 1% others (including one *N. lactamica* and other unidentified gram-negative bacilli).

The transformation results, in terms of the number giving a positive transformation to pro^+, are given in Table 1. It can be seen that false-positives amounted to some 14% of the total positives, an unacceptable level, but these were substantially all MG which occurred almost exclusively from throat swabs. If these could be eliminated, then all the positives would virtually be GC. A further 286 specimens consisting of 196 (68.5%) GC, 82 (28.7%) MG, and 8 (2.8%) others were assayed, identifying the MG from throat swabs by the aminopeptidase test (Table 2). The removal of the MG resulted in the transformation test giving no false-positives and over 99% reliability for known GC.

Transformation direct from swabs. Having shown that the transformation test could be reliable in identifying GC, it was now possible to ascertain whether the same degree of reliability

TABLE 2. Clinical isolates after the exclusion of the meningococcus giving positive or negative transformation of the *pro* gonococcus recipients to pro^+

Isolate	No. of specimens[a]	% Total	% Each species
GC[b]			
Transformation positive	195	95.6	99.5
Transformation negative	1	0.5	0.5
"Others"			
Transformation positive[c]	0		0
Transformation negative	8	3.9	100

[a] Total tested = 204.
[b] Identified by cultural, immunofluorescence, sugar, and aminopeptidase reactions.
[c] False-positives.

TABLE 3. Direct swab specimens giving positive or negative transformation of the *pro* gonococcus recipients to pro^+

Swab specimen[a]	Transformation			
	Positive		Negative	
	M[b]	W	M	W
Known GC	20 (51.3)[c]	34 (66.7)	19 (48.7)	17 (33.3)
Suspected GC	7		8	
Others	6	3	9	20

[a] Diagnosed by microscopy of a smear.
[b] M, Middlesex Hospital; W, Westminster Hospital.
[c] Numbers in parentheses indicate percentages.

could be attained when applied directly to the swab specimen without attempting to culture the organism. It was found, however, that the activities of the aminopeptidases present in the lysed swab sample, before it was heated to 60°C, appeared to be insufficient to allow their detection. It was necessary, therefore, to preclude specimens from throat swabs in this part of the survey. The clinic supplying the specimens made a diagnosis from another swab, and the specimens could be classified by their observations into the following three groups—(i) known GC, (ii) suspected GC, which included GC-like organisms in smears but were unculturable, and (iii) others, which included other *Neisseria* species and organisms which could mask the presence of GC. The results, in terms of the number giving a positive transformation to pro^+, are given in Table 3, and are subdivided into their origins. It can be seen that the reliability was reduced, with some 33% false-negatives from one source and some 49% from the other source. It is possible that the "quality" of the specimen varied, with one source giving a more consistently better specimen than the other. This idea was supported by the results obtained with 97 direct swab specimens from the Dreadnought Seamens' Hospital, Greenwich (not shown in the table), which included 20 specimens giving culturable GC, but no transformation-positive results were obtained with any of the specimens. However, it is of considerable interest that 7 out of the 15 "suspected GC" specimens and 9 out of the 38 "others" gave transformation-positive results.

Analysis of the other organisms. Table 4 gives a summary of the results obtained with 212 specimens found to be meningococci in the "others" category. MG made up 92% of these, of which 82% gave a transformation-positive reaction. Of the 17 non-MG specimens, only the one *N. lactamica* specimen transformed the GC recipient.

Elimination of MG. The results shown in Table 4 indicate the necessity for the reliable

TABLE 4. Nongonococcal isolates giving positive or negative transformation of the *pro* gonococcus recipients to pro^+

Nongonococcal isolate	No. of specimens[a]	% Total	% Each species
MG[b]		92.0	
Transformation positive	160	75.5	82.0
Transformation negative	35	16.5	18.0
Others		8.0	
Transformation positive	1[c]	0.5	6.0
Transformation negative	16	7.5	94.0

[a] Total tested = 212.
[b] Identified by the aminopeptidase profile.
[c] *N. lactamica*.

TABLE 5. Analysis of MG donor strains showing positive and negative transformations to pro^+ or leu^+ with *pro* and *leu* GC recipient strains

No. of donor MG strains	Source[a] of specimen	Aminopeptidase[b] profile of donor			Transformation with gonococcal recipient	
		G	OH	A	pro^-	leu^-
5	CSF	+	−	+	+	−
24	T	+	+	+	+	+
1	T	+	+	+	+	−
5	T	+	−	+	+	+
3	T	+	−	+	+	−
1	T	−	+	+	+	−

[a] CSF, Cerebrospinal fluid; T, throat.
[b] G, γ-L-glutamyl-4-nitroanilide; OH, L-4-hydroxyprolyl-β-naphthylamide; A, arginine-β-naphthylamide HCl.

identification of MG, since 82% of them were found to give false-positives. When the method used for the aminopeptidase profile was found to be unsuitable for application to direct swab specimen, a search was made for other auxotrophs as recipient organisms, since reports by Janik et al. (6) and Sparling et al. (9) have indicated that other auxotrophs integrate MG DNA at very much reduced frequencies. Two such auxotrophs were tested, namely, cys^- and leu^- *N. gonorrhoeae*.

(i) *cys* recipient strain. Catlin (3) and La Scolea and Young (7) reported that all MG strains tested from various sources in the United States were cys^+, whereas all GC strains were *cys*. However, of 740 clinical GC isolates screened for the possession of *pro, arg*, and *cys* characters, using the synthetic medium but omitting cysteine, glutathione, and cystine, only 71.5% (i.e., 529 isolates) were found to be *cys*, whereas the other characters were present in comparable proportions to those found in strains from the United States sources. Hence, the *cys* marker was unsuitable for use to differentiate between GC and MG.

(ii) *leu* recipient strain. DNA preparations from five specimens of *N. meningitidis* isolated from cerebrospinal fluid (kindly given by the Public Health Laboratories, Colindale, London), and which all gave the characteristic sugar and aminopeptidase profiles and were immunofluorescent negative for gonococcal antigens, were used as donors to both *pro* and *leu* gonococcus recipients. All transformed the *pro* recipient to pro^+ but not the *leu* recipient to leu^+. However, 34 specimens taken from throats gave interesting results. All showed characteristic sugar reactions and were immunofluorescent negative, but only eight gave the characteristic aminopeptidase profile for *N. meningitidis*, one gave a typical profile for *N. gonorrhoeae*, whereas the remaining 25 gave aminopeptidase activities which were a combination of both profiles. All 34 specimens transformed the *pro* recipient, and 24 of the mixed profile strains and 5 of the typical MG strains also transformed the *leu* recipient, whereas only the remaining 5 strains gave no transformation with the *leu*. All of the transformants possessed the aminopeptidase profile of a typical GC strain recipient. The results are summarized in Table 5.

DISCUSSION

It is clear from the results recorded in Table 2 that the DNA contained in the lysates of clinically isolated gonococci will transform a *pro* recipient gonococcus to pro^+ with 99.5% reliability. Also, apart from the meningococci, virtually no other organisms present in swabs from the same sites gave false-positives. The meningococci, however, gave some 85% of false-positives and must therefore be identified with a high degree of success by their aminopeptidase profile. Hence, it has been shown that the transformation test itself is capable of identifying the presence of gonococci with a very high degree of reliability with virtually no false-positives after identifying possible meningococci. This conclusion is supported by the results of Sarafian and Young (8) and Zubrzycki and Weinberger (10); the former authors showed that, of 169 isolates of *N. gonorrhoeae*, 90% gave positive transformation of a *pro* recipient, although some 50% of the *N. meningitidis* isolates gave positive results but at a lower sensitivity, whereas the latter authors distinguished 55 gonococcal isolates from other species of *Neisseria* by the use of *ts* mutants as recipients.

However, it is of crucial importance that the test should be as effective when applied directly to swabs without the need to culture the organisms. Although the results obtained so far showed too low a level of reliability, they did show promise. Since specimens likely to include

N. meningitidis were avoided, 99.5% of the positive results obtained can be confidently regarded as GC, and hence the seven positives classed as "suspected GC" and nine positives under "others" would in fact have been GC. Thus, the test was picking up the presence of GC in cases that were not culturable.

Two problems remain, however. Firstly, it is necessary to identify the MG that may be present because 82% of them would give false-positives. Since the aminopeptidase test in the form used was unsuitable for use on direct swab specimens, either it must be designed to work at a greater sensitivity, or a different approach must be used. A different auxotrophic marker may prove suitable since the reports of Janik et al. (6) and Sparling et al. (9) indicated that either some regions of the MG genome may be less acceptable to GC, or that certain auxotrophic markers are prevalent in GC rather than in MG. It is of interest to note, however, that whereas all of the GC tested in the United States have been *cys*, this was not the case in those studied from the London sources. The results obtained with the *leu* recipient were interesting, since it could be suggested that at least 24 of the 29 MG strains that did give positive transformation had some of their genome derived from GC. They possessed an aminopeptidase profile which, in addition to the characteristic MG profile, showed a positive reaction with L-4-hydroxyprolyl-β-naphthylamide as substrate which a typical MG would not split. This enzymatic ability may have been transferred from a GC in vivo before the organism was isolated, since Ison et al. (5) have recently shown the transfer by transformation of the gene specific for the *N*-γ-glutamylaminopeptidase from MG (in which it is normally present) to GC (in which it is normally absent). Whatever the explanation, it would seem that the *leu* recipient would also be unsuitable to differentiate clearly between GC and MG. More promising may be the use as recipients of *ts* mutants of both GC and MG as described by Zubrzycki et al. (10); whereas DNA from MG could transform the GC *ts* mutant, DNA from GC did not transform the MG *ts* mutant. Secondly, reliability of the test when applied to direct swabs must be improved. The lower reliability obtained may well have been due to the variability of the specimen. It was very likely that more than one swab was taken at the same time and that the ones presented for the transformation test may not have been equivalent in each case. A survey using first-taken swabs would check this point.

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