IDENTIFICATION OF PATHOGENIC NEISSERIAE BY GENETIC TRANSFORMATION

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DEVELOPMENTS in selective media and rapid identification procedures have considerably improved the cultural diagnosis of gonorrhoea (Jephcott and Rashid, 1978; Young, 1978*a*). When direct plating with immediate incubation is impracticable, several transport and growth systems such as Transgrow (Martin and Lester, 1971), Microcult GC (Willcox and John, 1976) and the biological environment chamber described by Martin and Jackson (1975) are now available, in addition to the conventional non-nutrient transport media such as Amies's modification of Stuart's medium (Amies, 1967).

Unfortunately, all of the above systems depend on maintaining the viability of *Neisseria gonorrhoeae* and in many localities transport-associated problems continue to impose limitations on their value. To overcome these problems it was recommended (World Health Organization, 1978) that methods for the detection of gonococcal components in the secretions of infected patients should be investigated.

Janik, Juni and Heym (1976) described a method for the detection of *N. gonorrhoeae* based on the ability of DNA samples from clinical isolates of gonococci to transform nutritional mutants of a particular strain of *N. gonorrhoeae*. At the time we wished to examine this technique in our own laboratory the proline auxotrophs used by Janik *et al.* (1976) could not be made available to us. Accordingly, we examined the feasibility of using a laboratory stock culture of *N. gonorrhoeae* strain F62, a strain known to require proline for growth in chemically defined medium (Catlin, 1973; Knapp and Holmes, 1975).

This paper reports our evaluation of the sensitivity and specificity of a genetic-transformation assay with this strain.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* strain F62 colony-type 2 was kindly supplied by Dr A. E. Jephcott, Public Health Laboratory, Myrtle Road, Bristol. The strain is auxotrophic for proline and was used as recipient in the genetic transformation assay. *N. gonorrhoeae* strain 9 was kindly supplied by Dr D. S. Kellogg, Centre for Disease Control, Atlanta, Ga, USA. Stock cultures of the following strains of *Neisseria* were supplied by the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London: *N. elongata* NCTC10660, *N. cinerea* NCTC10294, *N. catarrhalis* NCTC3622, *N. cuniculi* NCTC10297, *N. canis* NCTC10296, *N. pharyngis* var. *flavus* NCTC4590, *N. pharyngis* var. *siccus* NCTC4591, *N. denitrificans*

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NCTC10295, and *N. animalis* NCTC10212. *N. meningitidis* strains of serogroups A, B, C, D, E, 29E, W135, X and Z were originally obtained from the Neisseria Repository, Berkeley, California. The Oxford staphylococcus and a laboratory stock culture of *Pseudomonas aeruginosa* were obtained from the diagnostic section of this department. Subcultures of genital and oral isolates of neisseriae from patients attending the Department of Venereology, the Royal Infirmary, Edinburgh, were also obtained from the diagnostic laboratory; these included 169 strains of *N. gonorrhoeae*, 12 of *N. meningitidis*, three of *N. lactamica*, and one of *N. perflava*.

Gram staining and oxidase testing were done on all isolates. The clinical isolates were identified by the fluorescent-antibody test and the rapid carbohydrate-utilisation test as described by Young (1978a).

Culture media. Modified New York City (MNYC) medium (Young, 1978b) was used for subculture of isolates and for maintenance of *N. gonorrhoeae* strain 9. Stock strains of non-pathogenic neisseriae were maintained on MNYC medium lacking antibiotics.

Gonococcal genetic medium (GGM) (La Scole and Young, 1974) was used for growth and maintenance of *N. gonorrhoeae* strain F62 type 2 while it was auxotrophic for proline; the same medium lacking proline (GGM Pro⁻) was used to detect strain F62 when it became prototrophic for proline after transformation.

Preparation of transforming DNA. A sterile swab was charged with the growth from an overnight plate culture of the test organism. It was then placed in 0.5 ml of 0.025% (w/v) sodium dodecyl sulphate in standard saline citrate solution (0.15M sodium chloride, 0.015M sodium citrate) and agitated to remove bacteria. The resulting suspension was heated in a water bath at 65–68°C for 45–60 min. After heating, the crude DNA preparations were diluted with 0.3 ml of sterile distilled water to compensate for the increase in SDS concentration caused by evaporation.

Transformation assay. Three DNA samples, including DNA extracted from strain 9 as a control, were tested on a single GGM plate. The plate was divided into seven sections; two drops (approximately 0.04 ml) of a thick suspension (approximately 10¹⁰ colony-forming-units (c.f.u.)/ml) of strain F62 colony-type 2 in saline citrate were placed on each of three sections of the plate and one drop on a fourth section. Two drops of each of the three crude DNA preparations were deposited over the recipient cells in each of the three areas that had received two drops of F62. A drop of each of the DNA samples was placed on the remaining three sections as a sterility control. After incubation of the plate for 3 h at 37°C in a CO₂-enriched (10%) atmosphere, the contents of each section were streaked on to an identically marked plate of GGM Pro⁻ medium which was then incubated for 48 h as described above. The GGM plate was also incubated to check the viability of F62 and to monitor for contamination.

After incubation, the GGM Pro⁻ plates were examined with a Zeiss binocular stereoscopic microscope with substage lighting (Jephcott and Reyn, 1971). A positive result in the transformation assay was indicated by growth of several colonies (≥ 20 colonies) identical in size and appearance to the colonies of strain F62 growing on GGM. The results of the test specimens were compared with those obtained with *N. gonorrhoeae* strain 9. The section containing non-DNA-treated F62 streaked on to GGM Pro⁻ was a control to detect spontaneous revertant prototrophic cells.

Relationship of colony-type of recipient to the efficiency of transformation. Viable counts were made on thick suspensions of *N. gonorrhoeae* strain F62 colony-types 2 and 3 by the method of Miles, Misra and Irwin (1938); the dilutions were made in saline citrate and the colonies counted on clear GC agar (Kellogg *et al.*, 1963). A crude DNA preparation was made from *N. gonorrhoeae* strain 9. Hundredfold dilutions of the DNA preparation were made in saline citrate up to a dilution of 10¹² and assayed as already described with cells of colony-types 2 and 3 as recipients.

Assessment of sensitivity of the test procedure with *N. gonorrhoeae* strain 9 and *N. meningitidis* serogroup B. A crude DNA preparation was made from a thick suspension of *N. gonorrhoeae* strain 9: the number of bacteria present in the suspension was determined by the method of Miles *et al.*, (1938). Hundredfold dilutions of the crude DNA were made up to a dilution of 10¹². Each dilution was assayed as previously described. The procedure was repeated with *N. meningitidis* serogroup B as test organism.

Assessment of proportion of clinical isolates auxotrophic for proline. Eighty-four of the clinical isolates were subcultured on to GGM and GGM Pro⁻ media to test their requirement for proline.

Duration of storage of test organisms on swabs. Nineteen MNYC plates were seeded with *N. gonorrhoeae* strain 9. After overnight incubation, the growth on each plate was harvested with a sterile serum-coated swab (Exogen Ltd, Clydebank Industrial Estate, Beardmore Street, Clydebank G81 4SA, Scotland). Nine of the swabs were stored at 4°C, nine at room temperature and the remaining swab was used immediately in a transformation assay. The test organisms on the swabs, stored as described above, were assayed at intervals up to 68 days.

RESULTS

Colony type of recipient in relation to the efficiency of transformation

Viable counts showed that suspensions of strain F62 types 2 and 3 used as recipient contained approximately 10^{10} c.f.u./ml. With a series of DNA dilutions up to 10^{12} made from *N. gonorrhoeae* strain 9, strain F62 colony-type 2 gave a positive transformation result up to a dilution of 10^6 DNA whereas colony-type 3 gave a positive transformation result up to a dilution of 10^4 .

Stock strains

Apart from *N. gonorrhoeae* strain 9 and *N. meningitidis* strains of serogroups A, B, C, D, E, 29E, W135, X, Z, all of the remaining stock cultures, including the various other members of the family Neisseriaceae failed to transform *N. gonorrhoeae* strain F62.

Clinical isolates

Of the 169 clinical isolates of *N. gonorrhoeae*, 150 (88.8%) gave a positive transformation assay. All 12 of the meningococcal isolates tested were positive as were the three strains of *N. lactamica*. *N. perflava* gave a negative transformation result.

Assessment of proportion of clinical isolates auxotrophic for proline

Of 84 clinical isolates of gonococci tested, 73 gave a positive transformation assay. Eighteen of the isolates grew on GGM and on GGM Pro⁻, 58 failed to grow on either medium, 8 grew only on GGM, and none grew only on GGM Pro⁻. Of the 8 strains growing only on GGM, 6 (75%) gave a negative result in the transformation assay. All of the 18 strains that grew on both media gave a positive transformation result.

Sensitivity of transformation assay

The thick suspension of *N. gonorrhoeae* strain 9 contained 1.7×10^{10} c.f.u./ml and gave a positive transformation result up to a dilution of 10^6 . Because 0.1 ml of this suspension was placed in 0.9 ml of the SDS solution, the

crude DNA preparation was obtained from cells at a concentration of 1.7×10^9 c.f.u. Thus, 0.04 ml of the most concentrated sample of DNA tested in the transformation assay was derived from 6.8×10^7 c.f.u. and 0.04 ml of the maximum dilution that gave a positive result corresponded to 68 c.f.u.

The thick suspension of *N. meningitidis* serogroup B contained 4.7×10^9 c.f.u./ml and gave a positive transformation result up to a dilution of 10^2 . Because 0.1 ml of this suspension was placed in 0.9 ml of the SDS solution, the crude DNA preparation was obtained from cells at a concentration of 4.7×10^8 c.f.u. Thus, 0.04 ml of the most concentrated sample of DNA tested in the transformation assay corresponded to 1.9×10^7 c.f.u. and the maximum dilution that gave a positive result corresponded to 1.9×10^5 c.f.u.

Storage of test organisms on swabs

Swabs bearing test suspensions stored at 4°C and room temperature gave a positive result when tested at intervals up to 68 days. The effect of longer storage was not tested.

DISCUSSION

Our results confirm the findings of Janik *et al.* (1976) that genetic transformation can be used as a tool for the identification of *N. gonorrhoeae*. Although we used a different strain as recipient, the overall sensitivity of the technique is comparable. Janik *et al.* (1976) found that approximately 50 c.f.u. of donor cells were required to give a positive result compared with approximately 70 c.f.u. in our studies with *N. gonorrhoeae* strain F62. As Janik *et al.* (1976) found, colony-type 2 was more efficient than colony-type 3 in the uptake of DNA.

With the exception of *N. meningitidis* and *N. lactamica*, the transformation assay is specific for *N. gonorrhoeae*. A positive result was obtained with all 12 clinical meningococcal isolates and the stock cultures of the different meningococcal serogroups. These results are also similar to those of Janik *et al.* (1976) who found that DNA preparations from only a few neisseriae other than the gonococcus were able to transform their proline auxotrophs; these preparations were unable to transform a uracil and arginine auxotroph in the standard transformation assay conditions. The lack of specificity with regard to *N. meningitidis* would not obviate the value of the assay in detecting ano-genital gonorrhoea because meningococci are relatively rare in the urogenital tract and anal canal (Givan, Thomas and Johnston, 1977; Blackwell, Young and Bain, 1978) and the number required to give a positive transformation assay is approximately 1000-fold higher than when gonococci are used. However, in view of the positive results given by meningococci the genetic-transformation assay described would not be suitable for diagnosing pharyngeal gonorrhoea.

Naturally-occurring proline auxotrophs do not appear to limit the value of the assay greatly because approximately 90% of the clinical isolates gave a

positive transformation assay. In another locality Bawdon, Juni and Britt (1977) reported that 97% of 71 clinical isolates of *N. gonorrhoeae* gave a positive transformation result in an assay that used a uracil and arginine auxotroph. Proline requirement is probably the main reason for negative results in our survey because 6 of 8 (75%) strains that failed to grow on GGM Pro⁻ gave negative results whereas all of the 18 isolates that grew on both GGM and GGM Pro⁻ gave positive results. However our results with respect to the growth of isolates on GGM and GGM Pro⁻ do not seem to be very reliable because 58 of 84 (69.0%) fresh clinical isolates failed to grow on both media; clinical isolates probably need to be adapted to growth in the laboratory before giving reliable growth on a minimal medium such as GGM.

After prolonged storage of gonococci on swabs, a positive transformation result could still be obtained. Therefore the ability of the assay to detect non-viable gonococci would make this test of value in overcoming problems associated with the transport of specimens before cultivation by conventional methods. A trial is now under way to assess the value of this procedure in clinical practice.

SUMMARY

The detection of pathogenic neisseriae by genetic transformation of a naturally occurring proline auxotroph of *Neisseria gonorrhoeae* strain F62 is described. Of 169 clinical isolates of *N. gonorrhoeae*, approximately 90% gave a positive transformation assay. Twelve clinical isolates of *N. meningitidis* and stock cultures of the various meningococcal serogroups also gave a positive result. However, the sensitivity of the assay was found to be approximately 1000-fold lower with *N. meningitidis* as test organism. Eleven other members of the family *Neisseriaceae* failed to transform the recipient organism.

Although proline requirement did not appear to limit the value of the assay greatly, it probably was the main reason for negative results. The sensitivity of the assay and its ability to detect non-viable gonococci suggests that this method merits further investigation as a possible aid to diagnosis of gonococcal infection in special circumstances.

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