

## Laboratory methods in genitourinary medicine

# Methods of diagnosing gonorrhoea

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### Abstract

**Gonorrhoea is normally diagnosed presumptively by the presence of intracellular Gram-negative cocci on a Gram stain and confirmed by culture of the causative organism, *Neisseria gonorrhoeae*. Alternative methods have been evaluated extensively for the detection of gonococci in clinical specimens including immunological techniques such as ELISA and immunofluorescence, DNA probes, genetic transformation and the limulus lysate assay. Some of these tests have proved as sensitive and specific for the detection of gonorrhoea in symptomatic men as the Gram stain but offer no advantage in time or cost. In women, no test has been found that shows a sensitivity and specificity sufficiently adequate for clinical use. Culture in men and women remains the method of choice for diagnosis. In addition the need to obtain the infecting organism for antibiotic susceptibility testing has not been overcome. In contrast, the rapid identification of *N gonorrhoeae* can be achieved within four hours using either monoclonal antibodies or by the detection of preformed enzymes. New methods for both the detection and identification of *N gonorrhoeae* should be carefully evaluated particularly for use in cases of child and sexual abuse where medico-legal problems may arise.**

Gonorrhoea is a major cause of sexually transmitted infection with 25 898 cases of post-pubertal gonorrhoea reported from genitourinary medicine clinics in 1987 from England and Wales (CDSC, personal communication). The majority of infection is diagnosed at clinics for sexually transmitted infections and most of the remainder through general practitioners. Diagnosis of gonorrhoea needs to be rapid to enable appropriate therapy to be given and hence prevent transmission of the infecting organism. Ideally diagnosis would be made at the patient's first visit to the doctor and treatment given immediately.

### Microscopy and culture

In an attempt to achieve this the Gram stain is used as presumptive diagnosis for gonorrhoea. The presence of intracellular Gram-negative cocci are considered diagnostic of gonorrhoea. In symptomatic men the sensitivity (ie the number of patients with gonorrhoea giving a positive test) of the Gram stain with urethral smears is over 98%.<sup>1-3</sup> However, in smears from the urethra of asymptomatic men, from the rectum and from the cervix in women, the Gram stain has a sensitivity of 40-50% at best.<sup>1-4</sup> Despite its lack of sensitivity in these patients the Gram stain has been used extensively as a presumptive diagnosis for gonorrhoea because it is easy and quick to perform and when examined by experienced personnel has a high specificity (ie the number of patients without gonorrhoea giving a negative test). Culture for the causative organism, *N gonorrhoeae*, is considered necessary to confirm the diagnosis and is regarded as "the gold standard". Successful isolation of *N gonorrhoeae* requires both good specimen collection and transport and a suitable culture medium. The urethra in men and the endocervix in women are the primary sites for colonisation and should be sampled either with a loop or a swab made of material that is non-inhibitory to gonococci. Rectal and pharyngeal swabs should also be taken if anal or oral intercourse has taken place. It is ideal to inoculate specimens from any site directly on to isolation media which are kept in the clinic. The inoculated media should then be incubated at 36°C in 7% carbon dioxide until they are transported to the laboratory. At St Mary's Hospital, London we have used this system for many years and we find it gives a high isolation rate. However, it may not always be possible to plate the specimen directly. The specimen should then be taken on a swab and immersed in a suitable transport medium such as Stuart's or Amies' medium. Gonococci will survive in a transport medium particularly if stored refrigerated but should be sent to the laboratory as soon as possible. An enriched medium is necessary for growth of this fastidious organism and antibiotics should also be added to suppress the growth of other bacteria and yeasts found in anogenital sites. The medium used most commonly is GC agar base supplemented with lysed horse blood or IsoVitaleX, which contains essential amino-acids and glucose.<sup>5-7</sup> Antibiotics such as vancomycin, to inhibit Gram positive organisms, colistin and trimethoprim, to inhibit other Gram negative organisms and amphotericin, to prevent the growth of

yeasts, are usually added to the medium.<sup>8</sup> The sensitivity of culture can be affected by the presence of these antibiotics which may also inhibit some strains of *N gonorrhoeae*.<sup>9,10</sup> Some STD clinics use a medium both with and without the addition of antibiotics to overcome this problem. Non-selective media are suitable for use with urethral specimens from men where the gonococci in an infected patient are greater in number than the normal flora. However, in cervical and rectal specimens the large numbers of normal flora present often prevent isolation of the gonococci present at these sites. The sensitivity of culture is, nevertheless, regarded as approaching 95–100% in clinics and laboratories where there is experience in taking suitable specimens and expertise in isolation and identification procedures. In general practice or smaller clinics without a laboratory on site, delay in the transport of the specimen may influence the sensitivity.

### Detection of gonococci in clinical specimens

#### Immunological techniques

In recent years, newer techniques have been used to detect gonococcal antigen in clinical specimens and to identify the organism isolated after culture. The same basic principles have been applied to both of these applications. Tests that detect gonococcal antigen in a clinical sample have been studied to provide an alternative or replacement to the Gram stain and/or culture. The most widely evaluated has been the immunologically based tests. Antibodies,

both polyclonal raised in rabbits and monoclonal, considered specific for *N gonorrhoeae*, have been utilised in enzyme-linked (ELISA) or immunofluorescence assays. In such tests the clinical specimen is applied to a solid phase, either a tube or slide, to which the gonococcal antigen will adhere if it is present (fig 1). The specific antibody or mixture of antibodies is then added and will attach to any gonococcal antigen. The complexes formed are then detected by the addition of an antiglobulin linked to either an enzyme or fluorescein and subsequently visualised by the addition of a chromogenic substrate (fig 1a) or using a fluorescent microscope. In some instances the procedure has been shortened by conjugating the specific antibodies directly to an enzyme or fluorescein (fig 1b) eliminating the need for the antiglobulin step.

An ELISA using polyvalent antiserum has been tested extensively for the detection of gonococcal antigen (Gonozyme, Abbott Laboratories). In comparison with culture for *N gonorrhoeae*, this test has a high sensitivity, 87–100%, and specificity, 99.4–100%, when used in high-risk populations of symptomatic men but offers no advantage to the Gram stain.<sup>2,3,11–14</sup> In women, the test shows greater discrepancies between centres and in general shows a lower sensitivity, 60–100%, and specificity, 71–100%.<sup>3,11–18</sup> Gonozyme is not reliable as a test of cure as it will detect antigen present on non-viable gonococci. Neither is it suitable for rectal samples because of the contamination with large numbers of organisms some of which may cross-react with the antibodies. A direct immunofluorescence test for diagnosing gonorrhoea using a mixture of monoclonal antibodies was also found to be sensitive, 85%, and specific, 100%, in men, but the findings were similar to those of the Gram stain.<sup>19</sup> When tested in women the sensitivity, 72%, and specificity, 94%, were higher than the Gram stain but lower than culture.<sup>19</sup> In these immunologically based tests one of the major limiting factors is the antibodies used. Polyvalent antiserum has the advantage that it will be cross-reactive with many strains of *N gonorrhoeae* but has the disadvantage that it may also cross-react with other *Neisseria* spp particularly *N meningitidis*. Monoclonal antibodies offer a greater specificity but this in itself may limit the number of strains of *N gonorrhoeae* that will react. The monoclonal antibodies that have been used in gonococcal tests have been raised to epitopes on the major outer membrane protein, PI.<sup>20</sup> The reagents used in the detection and identification tests contain mixtures of these antibodies to enable as many strains as possible to be detected. However, until a single monoclonal antibody that reacts with a conserved epitope present on all strains is used there will always be the potential for new strains to appear that do not react with the mixtures.

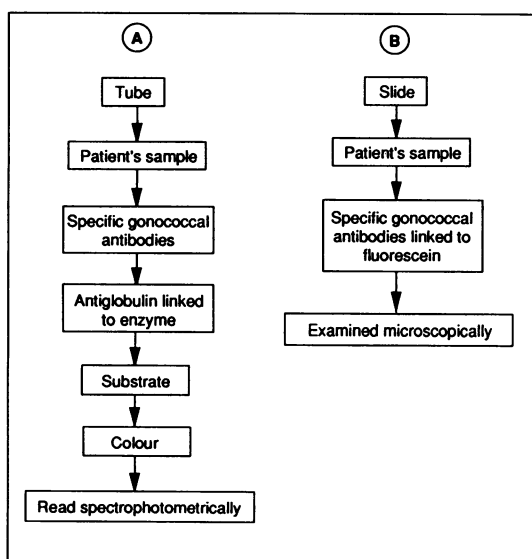


Figure 1 Principle of immunologically based tests for detection of gonococcal antigen.

### DNA probes

The use of DNA probes has also been given consideration for diagnosing gonorrhoea. In such tests a piece of DNA with sequences specific to *N gonorrhoeae* is used as a probe. The probe is labelled and mixed with a sample that may contain gonococcal DNA. If the same sequences are present in the sample the probe will stick (hybridise) and the detection system will give a positive result (fig 2). Initially the cryptic plasmid, which is present in the majority of strains of *N gonorrhoeae*<sup>21</sup> was used as the probe<sup>22</sup> and was radiolabelled with <sup>32</sup>P. A positive result was seen as blackening of a photographic film. The complete procedure could take up to three days. This test could never replace the Gram stain as a presumptive diagnostic test because it is time consuming, needs facilities to work with radioactive materials and a high level of expertise but it could have a role to play as an alternative or adjunct to culture particularly in asymptomatic patients. However, the premise on which this test is based is that the majority of gonococci carry the cryptic plasmid, hence giving a high sensitivity, and that no other species of bacteria carry the plasmid or have DNA which will show homology, hence giving a high specificity. However, both these assumptions have

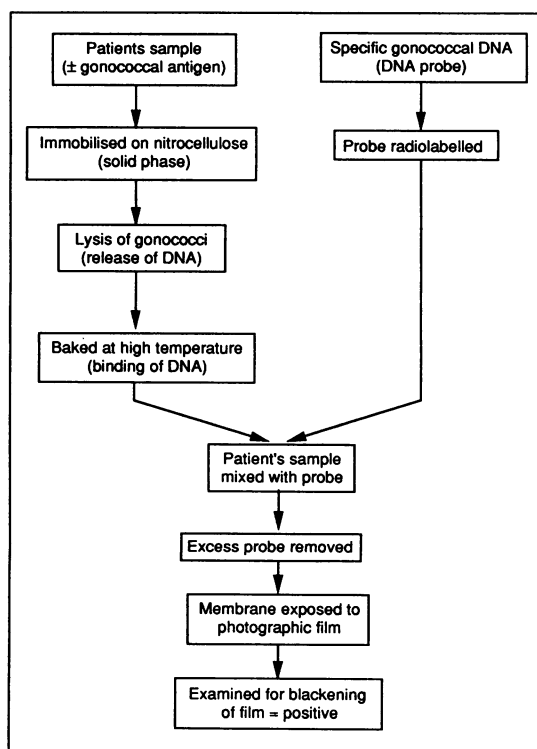


Figure 2 Principle of DNA probes for detection of gonococcal antigen.

proved to be partially incorrect. Some strains of *N gonorrhoeae* do not carry the cryptic plasmid<sup>23</sup> and the prevalence can be as high as 10% in certain geographical areas.<sup>24</sup> It is also true that a small number of strains of *N meningitidis* carry a plasmid which shows strong homology to the gonococcal cryptic plasmid.<sup>25</sup> Consequently in many populations the sensitivity and specificity of this plasmid probe is less than culture.

A different approach to the search for a specific piece of DNA is to use chromosomal DNA specific to *N gonorrhoeae* as a probe. The choice of the DNA sequence could prove difficult because *N gonorrhoeae* and *N meningitidis* share 80% homology between their genomes. Clones with DNA sequences specific for *N gonorrhoeae* have been chosen from a library of genomic DNA screened against strains of both *N gonorrhoeae* and *N meningitidis*. Using this approach Donegan *et al*<sup>26</sup> have isolated three DNA probes that are highly specific for *N gonorrhoeae* but display no cross-reactivity with *N meningitidis* or other members of the Neisseriaceae and show potential for use in diagnostic tests. Recently an oligonucleotide directed against rRNA specific to *N gonorrhoeae* was found to have 100% sensitivity and specificity against organisms which had already been isolated.<sup>27</sup> The development of probes such as these will enable this technology to have greater potential for use on clinical specimens for the diagnosis of gonorrhoea. Detection systems also need to be fully developed and evaluated before these tests will be used widely. The use of radiolabels requires time to obtain a result and is technically hazardous. Detection systems which have a chromogen as the label are currently available for the identification of *N gonorrhoeae* after isolation on selective media and give a result in 10–15 minutes.<sup>28,29</sup> Further evaluation of these tests will determine whether these DNA probes have a future for use with clinical specimens.

### Other techniques

There are reports of other techniques which have been evaluated for the detection of gonococcal antigen such as the use of genetic transformation<sup>30–32</sup> and the limulus lysate test.<sup>33</sup> These have proved to be technically demanding and time consuming and show little potential for adaption to a rapid diagnostic test.

### Problems associated with new tests

Despite the potential of the tests described above for the rapid and accurate diagnosis of gonorrhoea, they have not been used in the United Kingdom. In part, this has been the result of specific problems of the different techniques, already mentioned. However, they also share a number of problems which have prevented their widespread use. Firstly, the newer tests are all more costly and in many instances require

either greater expertise or more expensive equipment than the Gram stain. Secondly, it has proved difficult to produce a test that can give a result as quickly as the Gram stain. ELISA and DNA based tests require the specimen to be batch tested in the laboratory. The immunofluorescence test can be prepared and read in a clinic but it would take considerably longer than the Gram stain. Any replacement for the Gram stain will need to be as rapid with a considerable improvement in sensitivity. Thirdly, in women the number of gonococci is often low and are present in cervical mucus containing many other bacteria. The number of organisms, reported to be as low as  $10^2$ ,<sup>34</sup> has presented a problem for DNA probes and even ELISA tests. The presence of cervical mucus appears to prevent the removal of the gonococci from a swab to a slide and has kept the sensitivity of immunofluorescence tests low even when strains isolated from these patients are known to be reactive with the antibodies used. It is probable that this problem also contributes to the low sensitivity of the Gram stain in women. Finally, many of these new tests have shown good sensitivity and specificity but they must also be evaluated by their predictive value. The predictive value for a positive test (that is, the ability of a positive test to indicate gonorrhoea) and for a negative test (that is, the ability of a negative test to indicate the absence of gonorrhoea) are affected by the prevalence of gonorrhoea in the population tested. Tests with a sensitivity and specificity of more than 90% can still result in alarmingly high numbers

of false positives in low prevalence populations.<sup>14 18</sup> For example, in a population of 10 000 where the prevalence of gonorrhoea, as defined by culture, is 10%, a test with a sensitivity and specificity of 95% would result in 50 false positives (fig 3A). The predictive values for a positive and negative test would be 95% and 99% respectively.<sup>35</sup> However, in the same population with the same test but where the prevalence is 1%, the number of false positives would be 495 and the predictive values for a positive and negative test would be 19% and 99% respectively. The presence of false positives would necessitate recalling patients, repeat examination which would be particularly distressing for women and all the associated psychological, social and medico-legal problems of the possible presence of a sexually transmitted infection. In this regard any test for a sexually transmitted infection needs to have a high specificity in addition or even at the expense of a high sensitivity, a constraint not always encountered in other areas of microbiology.

The changing epidemiology of gonorrhoea has also affected the commercial market for any new diagnostic test. The number of cases of gonorrhoea has fallen since 1984 from a total of approximately 53 802 cases<sup>36</sup> to 25 898 cases in 1987 (CDSC, personal communication) in England and Wales. The fall has been attributed to changes in sexual practice linked to increased education and fear of infection with the Human Immunodeficiency Virus (HIV). At St Mary's Hospital, London, where a large number of patients infected with HIV are treated, we have seen a fall from 3500 cases in 1984 to 900 cases in 1989. Although this decline is greatest in the homosexual population there is also a dramatic fall in heterosexual gonorrhoea. Recent figures suggest that the number of cases of gonorrhoea at St Mary's Hospital is rising slightly but it is unlikely that we will return to the numbers of cases seen before the advent of the HIV epidemic. In contrast to this decline in gonococcal infection, there has been an increase in the number of strains of *N gonorrhoeae* exhibiting antibiotic resistance.<sup>7 37 38</sup> This is the result of the ability of the organism to adapt and evolve mechanisms of resistance and from the importation of strains from the developing world where both the number of cases and the level of resistance to antibiotics remains high. Consequently, two factors have influenced the market for any new test in the United Kingdom, the reduced number of tests for gonorrhoea that need to be performed and the need to culture the organism to enable antibiotic sensitivity testing to be performed. Tests that will detect antibiotic resistance in the clinical sample and therefore eliminate the need to culture the organism have been described but only detect plasmid-mediated resistance.<sup>39-41</sup> Chromosomally-mediated antibiotic resistance in *N gonorrhoeae* is current at least as great a problem as

		(A) Prevalence = 10%	
		Culture Positive	Culture Negative
New test	Positive	950	50*
	Negative	50	8550
		1000	9000
		(B) Prevalence = 1%	
		Culture Positive	Culture Negative
New test	Positive	95	495*
	Negative	5	9405
		100	9900

\*False positives

Figure 3 Effect of prevalence of gonorrhoea on the efficiency of a new test for diagnosis with a sensitivity and specificity of 95%.

plasmid-mediated resistance and therefore there is still a need to culture the organism and perform susceptibility testing. To my knowledge, there are very few, if any, centres in the United Kingdom using the techniques described above for the detection of antigen for the routine diagnosis of gonorrhoea. It is the identification of organisms grown by culture that has utilised these newer technologies more successfully and these have become widely used and accepted alternatives to the conventional tests.

### Identification of *N gonorrhoeae*

#### *Conventional tests*

Identification tests for *N gonorrhoeae* fall into two categories, screening tests to establish the presence of *Neisseria* spp and tests to differentiate *N gonorrhoeae* from *N meningitidis* and non-pathogenic species. The Gram stain and oxidase reaction are the screening tests used to establish that any colonies on the primary isolation medium are oxidase positive, Gram negative cocci. Both these tests are quick and easy to perform. However, identification of the species, *N gonorrhoeae* is more difficult and time consuming. Carbohydrate utilisation tests have been used historically, *N gonorrhoeae* differing from other species in that it produces acid only from glucose. Conventional tests have used an agar base capable of supporting the growth of *Neisseria* spp with added carbohydrate.<sup>42</sup> These media require a pure growth for inoculation and require 24 hours incubation at 37°C before a result can be obtained. Subsequent modifications of this technique using liquid media<sup>43,44</sup> or commercially available kits<sup>45-47</sup> have allowed identification within one to four hours but a heavy pure culture is still required and, therefore, identification is still delayed for 24 hours after isolation of the organism. Carbohydrate utilisation has been considered the definitive means of identifying *N gonorrhoeae* and has been the reference test in medico-legal cases.

#### *Use of monoclonal antibodies*

The newer technologies have broadened the spectrum of tests available for identification of *Neisseria* spp. The most successful of these has been the use of monoclonal antibodies in tests that can identify colonies of *N gonorrhoeae* from a primary isolation medium and do not require a pure culture. Two tests are widely available in the United Kingdom, Phadebact Monoclonal GC OMNI Test (Pharmacia, Sweden) and GC Microtrak (Syva Company, USA). Both of these reagents contain a mixture of monoclonal antibodies raised to epitopes on the two types of the major outer membrane protein, PI, to give a broad cross-reactivity. The tests differ in that the Phadebact reagent contains monoclonal antibodies which are linked to staphylococcal Protein A and are

used in a coagglutination test whereas in the GC Microtrak reagent the antibodies are linked to fluorescein and the reagent is used on smears which are examined by a fluorescent microscope. These reagents give a positive result with *N gonorrhoeae* and are negative with all other species of *Neisseria*. Both tests have been shown to be highly sensitive and specific<sup>47-51</sup> and in many laboratories have replaced carbohydrate utilisation tests because they are easy to use and a result is obtained on the same day as the organism is isolated. Some strains have been described which are negative with one or other of these reagents but, as described above, this will always be a problem with reagents using a mixture of antibodies. We have found these reagents to be highly specific (that is, giving few false positives) and that any unexpected negatives can easily be identified by biochemical methods.

#### *Use of preformed enzymes*

Rapid identification using enzyme profiles often in combination with carbohydrate utilisation is also available mostly as kits.<sup>45,47,52</sup> These tests are mainly based on the work of D'Amato *et al*<sup>53</sup> who used chromogenic substrates to detect a range of aminopeptidases. This approach is useful for the identification of *N meningitidis*, particularly those strains which do not utilise maltose and hence appear as gonococci. The gamma-glytamyl-aminopeptidase test is positive for *N meningitidis* but is always negative with strains of *N gonorrhoeae*. Unlike the immunological identification, these tests attempt to differentiate the species of the genus *Neisseria*, for instance between *N gonorrhoeae*, *N meningitidis* and *N lactamica*. This approach to rapid identification has not been as widely accepted as the use of monoclonal antibodies possibly because it generally needs a heavy pure growth for inoculation which increases the time of identification and this level of identification is not required by most laboratories. It is sufficient in many cases to ensure the correct identification of *N gonorrhoeae* and to eliminate other *Neisseria* spp without further identification.

#### *DNA probes*

In a similar manner to the detection of *N gonorrhoeae* in clinical samples, DNA probes have not been used for the identification of *N gonorrhoeae*. Although these tests may offer a greater specificity than immunological tests it is only recently that the major obstacles of the radioactive label and the time needed to complete the test have been addressed.<sup>28,29</sup>

#### *Medico-legal implications*

It must be remembered that the correct identification of *N gonorrhoeae* is particularly important in cases of sexual and child abuse. It is unlikely that most laboratories have the facilities available routinely to

perform a full biochemical identification as described by Knapp<sup>54</sup> which uses a panel of eight tests. It would therefore seem sensible to perform more than one of the identification tests described above to confirm *N gonorrhoeae* if the isolates are known to be from cases with medico-legal implications.

Immunological and DNA techniques have been applied to both the detection of gonococci in a clinical sample and to identification of the isolated organism but only the latter of these has been successful. Despite the failure to find an alternative to the Gram stain and culture for the diagnosis of gonorrhoea the search for a rapid, sensitive test still continues. This is somewhat surprising in that any of the newer tests will be considerably more expensive to perform. However, a test that can diagnose gonorrhoea while the patient is still present could be cost-effective because it would enable suitable therapy to be given immediately and prevent recall of the patient. Any test that is to replace culture entirely will need to predict the antibiotic susceptibility of the infecting organism. Resistance to penicillin has become such a problem worldwide that the World Health Organisation have recently changed their recommendations for first line therapy from penicillin to ceftriaxone, spectinomycin or ciprofloxacin.<sup>55</sup> All current methods for the detection of resistance to these antibiotics require a viable culture of the organism. The rapid identification of *N gonorrhoeae* appears to be simpler but the limitations of each test should be considered carefully, particularly if it is to be used alone, when the organism has been isolated from a case of child or sexual abuse.

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