ACTA MEDICA LITUANICA. 2007. Vol. 14. No. 2. P. 123–134

Guidelines for laboratory diagnosis of *Neisseria gonorrhoeae* in East-European countries.

Part 2. Culture, non-culture methods, determination of antibiotic resistance, and quality assurance

Alevtina Savicheva[1], Evgenij Sokolovsky[2], Natalia Frigo[3], Tanya Priputnevich[3], Tatjana Brilene[4], Judith Deák[5], Ron Ballard[6], Cathy Ison[7], Anders Hallén[8], Marius Domeika[9*], Magnus Unemo[10]

[1] *Microbiology Laboratory, DO Ott Institute for Obstetrics and Gynecology, RAMS, St. Petersburg, Russia*

[2] *Department of Dermatology and Venereology, Pavlov State Medical University, St. Petersburg, Russia*

[3] *Microbiology Laboratory, Central Institute for Skin and Venereal Diseases, Moscow, Russia*

[4] *Department of Microbiology, University of Tartu, Tartu, Estonia*

[5] *Department of Microbiology, University of Szeged, Szeged, Hungary*

[6] *National Centre for HIV, STD, and TB prevention, Centers for Disease Control and Prevention (CDC), Atlanta, USA*

[7] *Sexually Transmitted Bacteria Reference Laboratory, Health Protection Agency (HPA), London, United Kingdom*

[8] *Department of Dermatology and Venereology, Uppsala University Hospital, Uppsala, Sweden*

[9] *Department of Medical Sciences, Uppsala University, Uppsala, Sweden*

[10] *Department of Clinical Microbiology, Örebro University Hospital, Örebro, Sweden*

INTRODUCTION

The present guidelines aim to provide comprehensive and precise information regarding the sexually transmitted infection (STI) gonorrhoea and laboratory diagnosis of the aetiological agent, i.e. *Neisseria gonorrhoeae* (the gonococcus), in East-European countries. The recommendations contain important and crucial information for both physicians and laboratory staff working with STIs and/or STI-related issues. For the different East-European countries, minor national adjustments of the present guidelines may be needed to oblige the requirements, lack of accessibility of, for example, some chemical reagents and equipment, and laws in each specific country. For information regarding gonorrhoea, sampling and transportation of clinical specimens, and microscopy for diagnosis, see Guidelines for laboratory diagnosis of *Neisseria gonorrhoeae* in East-European countries. Part 1 (Acta Medica Lituanica. 2007; 14(1)).

The present guidelines were written on behalf of Sexual and Reproductive Health and Rights (SRHR) Network, STI Diagnostic Group, which is supported by grants from the East Europe Committee of the Swedish Health Care Community, Swedish International Development Cooperation Agency (SIDA). Project coordinator Marius Domeika.

* Corresponding author. E-mail: marius.domeika@medsci.uu.se

European Union's case definition of gonorrhoea:

Clinical description: Clinical picture compatible with gonorrhoea, e.g., urethritis, cervicitis, or salpingitis.

Laboratory criteria for diagnosis:

– Isolation of *Neisseria gonorrhoeae* from a clinical specimen

– Detection of *N. gonorrhoeae* antigen or nucleic acid

– Demonstration of Gram-negative intracellular diplococci in an urethral smear from a male.

CULTURE OF *N. GONORRHOEAE*

In addition to microscopy of stained specimens, culture of *N. gonorrhoeae* should always be conducted:

- in children, as there are a great number of non-pathogenic commensal Neisseria species, especially in the oral cavity, pharynx and even genitals;

• if possible, for a reliable diagnosis in women due to the low diagnostic sensitivity and suboptimal specificity of stained urogenital samples used for diagnostics;

• in patients with early, asymptomatic and extragenital gonorrhoea (pharynx, rectum, conjunctiva, etc.);

• in sexual contacts of patients with confirmed gonorrhoea, whose microscopic examination failed to detect gonococci;

• in patients with confirmed gonorrhoea after completion of treatment (not earlier than in 8–10 days) and when withdrawing them from registration;

• for definitive diagnosis of gonorrhoea;

• to be able to determine antibiotic susceptibility of the *N. gonorrhoeae* strain;

• if phenotypic and/or genotypic characterisation of the *N. gonorrhoeae* strain will be performed;

• in cases of sexual abuse or other medico-legal purposes, if requested by investigating authorities and/or forensic experts.

Following sampling, culture results should usually be issued in 2–3 days, but if isolation of pure culture, definitive species identification and antibiotic susceptibility testing are required – in some cases it will take some additional day.

Principle of the method

Isolation of *N. gonorrhoeae* on culture media with subsequent morphological, biochemical, and species confirmative characterisation.

Advantages of culture for *N. gonorrhoeae* diagnostics:

• high sensitivity;

• high specificity;

• suitable for most types of specimens;

• inexpensive in comparison with NAATs;

• allows antibiotic susceptibility testing and phenotypic and/or genotypic characterisation.

Disadvantages of culture for *N. gonorrhoeae* diagnostics:

• lower sensitivity under non-optimised conditions;

• lower specificity under non-optimised conditions;

• time-consuming in comparison with microscopy and DNA / RNA-based methods, e. g., NAATs;

• more expensive than microscopy, however, microscopy is not an alternative for definitive and species-confirmed diagnosis of *N. gonorrhoeae* (see below).

Sampling

(see Guidelines for laboratory diagnosis of *Neisseria gonorrhoeae* in East-European countries, Part 1).

Transportation of samples

(see Guidelines for laboratory diagnosis of *Neisseria gonorrhoeae* in East-European countries, Part 1).

Since gonococci are sensitive to drying, extreme temperatures, and oxidation effects, swabs sampled from a patient are ideally inoculated directly on culture media. If this is not possible, appropriate transport media (see Guidelines for laboratory diagnosis of *Neisseria gonorrhoeae* in East-European countries, Part 1) must be used. Inoculated non-nutritive transport tubes should be placed in a refrigerator. Samples should be delivered to the laboratory as soon as possible following inoculation of the transportation medium and optimally inoculated on culture medium within 24 hours (maximum 48 hours). The transportation temperature should not be less than 18 °C.

Culture media

Gonococci are very fastidious bacteria with respect to, among many other things, the composition of the culture media. Highly nutritious media that contain a certain concentration of essential amino acids such as cysteine, purines and pyrimidines, as well as a usable energy source (i. e. glucose, pyruvate, or lactate) are needed to be able to culture all divergent gonococcal strains. In order to improve the identification of gonococci, antibiotics suppressing the growth of contaminating bacteria and fungi are added to the culture media. Such supplements include, e. g., lincomycin and/or vancomycin; colistin; nystatin, anisomycin or amphotericin B; and trimethoprim. However, some rare gonococcal strains can be susceptible to the included concentrations of vancomycin and/or trimethoprim.

Accordingly, for isolation of pathogenic Neisseria species, i. e. *N. gonorrhoeae* and *N. meningitidis*, a selective culture medium such as modified Thayer-Martin (MTM), Martin-Lewis (ML), New York City (NYC), or GC-Lect medium (or selective culture medium that is comparable, validated and certified for this purpose in the specific country) is used. All these media contain accurate concentrations of growth inhibitors for accompanying saprophyte microflora (see above). Of course, it is necessary to use effective selective culture media that in addition are certified in the specific country for culture of gonococci. Ideally, also a non-selective culture medium, especially for specimens obtained from less contaminated sampling sites, is used to complement the selective medium and also to identify the rare *N. gonorrhoeae* strains that are susceptible to vancomycin and/or trimethoprim. However, the prevalence of these strains is negligible in most countries. Various commercially available preprepared culture media exist, which may significantly vary in performance regarding growth ability of gonococci and suppression of other Neisseria species and contaminants.

Toxic factors

A culture of gonococci can be affected by various factors, e. g., treatment of the patient with antimicrobials as well as local application of gynaecological lubricants, irrigations, and spermicidal agents. Cotton, calcium alginate, and dacron swabs used for sampling can all be toxic for gonococci. This is due to the fact that unsaturated fatty acids in cotton fibres, chlorine-based bleaching agents, resin in wooden shafts, and glue used to connect swabs to the shaft may be toxic for gonococci. Consequently, direct inoculation on culture plates is ideal for reducing the time of exposure to possible toxic substances. Otherwise, the choice of appropriate swabs may be crucial, and charcoal-coated swabs should be used if charcoal is not included in the transportation medium. Ingredients such as starch, charcoal and blood of accurate transportation media can act as adsorbing agents and therefore increase the capacity of gonococci to survive during the transport.

Equipment and reagents required for culture and species confirmation of *N. gonorrhoeae*:

- plates with selective culture medium and, optimally, also non-selective agar plates;
- CO_2 incubator at 37 °C **or** candle (ideally, made of white wax or bee's wax) extinction jar and ordinary incubator at 37 °C;
- glass slides;
- chemical reagents and equipment for Gram's stain;
- microscope;
- sterile bacteriological loops;
- oxidase reagent (1% aqueous solution of tetramethyl-*p*-phenylendiamine dihydrochloride);
- assays for definitive species confirmation of *N. gonorrhoeae*.

Culture performance

Cultivation of *N. gonorrhoeae* using agar plates (Petri dishes)

The sample should be immediately inoculated on 90 mm or 100 mm diameter plates containing a minimum of 20 and 25 ml medium per dish, respectively. If it is not possible to use these large plates, 60 mm diameter plates containing medium with a depth of at least 4–4.5 mm may be used. However, it is not acceptable to utilize tubes for culture. During preparation of culture agar plates, to remove condensate (excessive moisture) after the medium has been poured and solidified in plates, the plates are placed inverted with the lids off in an incubator at 36 ± 1 °C for 1 hour or left overnight at ambient temperature. Overdrying the medium should always be avoided as this will result in reduced gonococcal growth. Prepared medium plates can usually be stored in a refrigerator at 6 ± 2 °C for at least up to 3 weeks in clean and sealed air-tight plastic bags. However, eventual instructions of the manufacturer have to be followed. Usage of the plates beyond the expiry date reduces the sensitivity of gonococcal cultivation. Prior to inoculation the plates should, ideally, be warmed to a temperature of 36 ± 1 °C.

Different clinical samples obtained from a patient should be inoculated on different plates. However, if large plates are used, i. e. 90 mm or 100 mm in diameter, material from the urethra and the endocervical canal of women can be inoculated on the same plate provided that the different sectors are accordingly marked. Material from the urethra of men must be inoculated on an individual plate.

The sampled material is applied, using a swab, on the agar surface equal to approximately one quarter of the medium surface area of a plate or in a sector of a plate. Afterwards the material should be spread over the remaining area of the culture medium surface using a sterile bacteriological loop with dashed movements in 3–4 different directions in order to identify individual colonies. The plates are then placed in an incubator containing 5 ± 2% CO_2, approximately 70% humidity, at a temperature of 36 ± 1 °C. A candle extinction jar, with e. g. moistened cotton wool to receive adequate humidity, placed into an incubator at 36 ± 1 °C is also acceptable. Plates are examined following 18–24 hours of incubation and again after 48 hours.

- If there are no signs of growth following 72 hours of incubation, observations are stopped and the sample is considered as negative.
- If suspicious *N. gonorrhoeae* colonies are detected, presumptive identification is carried out (see below).
- If necessary, species confirmatory identification for definitive diagnosis of *N. gonorrhoeae* (see below) is subsequently performed.

Cultivation of *N. gonorrhoeae* using commercially available culture systems (e. g., Biocult) can be performed as follows:

- allow the agar container to achieve room temperature;
- carefully, trying not to damage the agar slide, remove this from the container;
- inoculate the clinical specimen on the agar slide by rotating the sample brush / swab (a separate side of the slide should be used for each sample);
- sterile loops must be used for spreading the material;
- using sterile forceps, one CO_2-generating tablet should be aseptically removed and placed in the container;
- the moist atmosphere inside the container will cause release of gas from the CO_2-generating tablet, which is necessary for promoting gonococcal growth;
- slides containing inoculated clinical material should be placed into the container without touching the tablet;
- the container should be accurately marked;
- incubate at 36 ± 1 °C for 48 hours.

If suspicious gonococcal colonies are detected, presumptive identification is carried out (see below). If necessary, species confirmatory identification for the definitive diagnosis of *N. gonorrhoeae* is subsequently performed (see below). In comparison with culture on large medium plates (i. e., 90 mm in diameter), containing an appropriate culture medium, the growth ability of *N. gonorrhoeae* on Biocult slides is usually significantly lower.

Identification of *N. gonorrhoeae*

Presumptive identification of *N. gonorrhoeae* is based on the following:

- identification of characteristic colonies of *N. gonorrhoeae*;
- rapid positive oxidase test;
- identification of typical Gram-negative diplococci in material from suspicious colonies.

Identification of characteristic N. gonorrhoeae colonies

Following 18–24 hours of incubation, typical gonococcal colonies may appear as grey to white in colour, transparent to opaque, convex to flat, and having a diameter of approximately 0.5–1.0 mm. If further incubated, the colonies can increase in size to 3.0 mm and become less smooth and more flat. Frequently, different colony types of the same gonococcal strain or colonies of different bacterial species can be identified on the same plate. Great difficulties may arise in identification of gonococcal colonies in cultures, especially from the oropharynx, as in this case also meningococcal and non-pathogenic Neisseria species may be isolated, whose colonies are similar to those of gonococci. The size, colour, morphology and consistency of the colonies of all these species differ when using different culture media. A definitive diagnosis of *N. gonorrhoeae* can only be provided by further testing of the colonies, using a sugar utilization test or other species confirmatory tests (see below).

Application of oxidase test

Detection of oxidase-positive Gram-negative diplococci is considered sufficient for their presumptive identification as *N. gonorrhoeae* in routine diagnostics.

Identification of cytochrome-*c* oxidase is frequently performed using one of the following methods:

• a drop of oxidase reagent (tetramethyl-*p*-phenylenediamine dihydrochloride, 1% aqueous solution) is applied on a suspicious colony. A rapid, i. e. usually within 5–10 seconds, change of the reagent colour to deep blue-violet, which is maintained for over 30 seconds, is considered a positive test. Absence of colour change indicates a negative test. The reagent used in the oxidase test kills gonococci, therefore further investigation of these colonies requiring viable bacteria will be impossible. Consequently, it is recommended to apply instead a few drops of the reagent on blotting (filter) paper and conduct the test on the paper by transferring suspicious colonies with a sterile loop. The oxidase test is highly sensitive, but it is not specific for *N. gonorrhoeae* or even Neisseria species. Other bacteria that contain cytochromic oxidases may be oxidase-positive, and therefore all oxidase-positive cultures should be Gram-stained and microscopically examined;

• commercially available oxidase reagents supplied in crushable glass ampoules, e. g., BACTIDROP oxidase, can also be used as described above;

• commercial discs or strips impregnated with, e. g., dimethyl-*p*-phenylenediamine hydrochloride are also available. A single colony is applied on a disc or strip, eventually pre-saturated with distilled water, using a sterile loop. A deep-purple or blue colouration appearing in 10–20 seconds denotes a positive oxidase reaction. Absence of colour change indicates a negative test.

Attention!

• To avoid false-positive reactions, steel or nickel-chromium wire loops should not be used in the oxidase test, as surface oxidation of these metals may occur. Sterile platinum, plastic or glass loops are recommended;

• observance of the time interval of 10–30 seconds is important.

Gram staining of bacterial material from suspicious colonies

For microscopic examination with Gram's stain, the material should be taken from single suspicious colonies, mixed with a small drop of physiological saline solution on a glass slide, air-dried, fixed in a burner flame and stained (see Guidelines for Laboratory Diagnosis of *Neisseria gonorrhoeae* in East-European Countries, Part 1). The morphology, arrangement and colour of bacterial cells should be considered. Following 18–24 hours of cultivation, gonococci can be seen as clusters of Gram-negative (pink-red) typical diplococci.

In many cases it is difficult to interpret older cultures (≥48 hours), as a large quantity of swollen or completely lysed bacterial cells may be present. As the culture grows older, the polymorphic nature of gonococci increases.

Attention!

In primary cultures, in addition to Neisseria species, there may be colonies of, e. g., staphylococci and/or streptococci, which may easily lose their initial blue-violet colour, especially during a too long discoloration with alcohol, and acquire a pink-red colour. If this is the case, they can be erroneously identified as Gram-negative gonococci. Especially in preparations from young colonies, some of the gonococcal cells may be arranged as tetrads in the microscopic examination.

Examined microscopic slides containing Gram-negative diplococci from culture should be kept in the laboratory for a period of 3 months.

Species confirmatory identification

This is performed in the case of detection of oxidase-positive, Gram-negative diplococci to provide a definitive diagnosis of *N. gonorrhoeae*, especially for isolates obtained from extragenital sites, but also for all *N. gonorrhoeae* that are to be further characterised by phenotypic typing such as serological, genetic typing, and/or antimicrobial susceptibility testing. In addition, optimally for a highly sensitive and specific laboratory diagnosis of *N. gonorrhoeae*, at least two species confirmative assays, based on different principles, are utilized.

The following tests can be applied for definitive species confirmation of *N. gonorrhoeae*:

• sugar utilization and/or chromogenic enzyme-substrate tests;

• immunological tests (e. g., direct immunofluorescence, co-agglutination);

• DNA / RNA-based methods (e. g. NAATs).

Sugar utilization and/or chromogenic enzyme-substrate tests

The sugar utilization of *N. gonorrhoeae* should be studied using a pure gonococcal culture and enables identification of *N. gonorrhoeae* and differentiation of other Neisseria and similar species. The reactions may be false-positive owing to contamination of the gonococcal cultures with other bacteria and false-negative if gonococci have been cultivated for more than 24 hours (owing to autolysis) or if some inhibitory substances have been transferred from the primary selective culture plate. To prevent these scenarios, it is necessary to create a pure gonococcal culture by subcultivation of single, typical colonies from the primary culture plate on new plates of a non-selective medium (e. g., chocolate agar) for 18–24 hours.

The sugar utilization of *N. gonorrhoeae* may be studied in growth-dependent and/or growth-independent tests. In growth-dependent tests, sugars and other chemical reagents (e. g., pH indicator such as phenol red) are included in the culture test medium where gonococcal growth occurs. Growth-independent tests are internationally more widely used and give an opportunity to achieve a quicker (within one to four hours), cheaper, more clear-cut and equally specific result. Thus, the growth-independent tests study preformed enzymes of *N. gonorrhoeae* in a heavy inoculum of pure colonies. However, the use of reagent-grade sugars is crucial. There are various in-house and commercial assays available for studying the sugar utilization and biochemical activity of *N. gonorrhoeae*, which may differ in sensitivity and specificity. Sugar utilization reactions are usually identified as changes in medium colour; e. g., if phenol red is used as a pH indicator, a change from red to yellow due to the produced acidic metabolites is considered a positive test. Chromogenic enzyme-substrate tests that identify presence of other specific enzymes (*o*-nitrophenyl-β-galactosidase (ONPG), γ-glutamyl aminopeptidase (GLU-AMP), prolyliminopeptidase (PIP; hydroxyproline aminopeptidase (HPA); proline aminopeptidase (Pro-AP)) are intended mainly for a rapid differentiation of *N. gonorrhoeae* from other species that may grow on the selective culture medium, i. e. *N. meningitidis*, *N. lactamica* and some strains of *N. cinerea* and *Moraxella catarrhalis*. Substrate discs or tablets for detection of these enzymes are available from several manufacturers. Colour changes from colourless to yellow

Table 1. **Sugar utilization and enzyme production of different Neisseria species and other oxidase-positive species such as *Moraxella catarrhalis* and *Kingella denitrificans***

Species	Biochemical activity							
	Glucose	Maltose	Lactose	Saccharose (Sucrose)	Fructose (Levulose)	ONPG*	GLU-AMP**	PIP***
N. gonorrhoeae	+	–	–	–	–	–	–	+
N. meningitidis	+	+	–	–	–	–	+	(+/)–
N. lactamica	+	+	+	–	–	+	–	+
N. cinerea	(+/)–	–	–	–	–	–	–	+
N. polysaccharea	+	+	–	–	–	–	–	+
N. sicca	+	+	–	+	+			
N. mucosa	+	+	–	+	+			
*N. subflava***	+	+	–	+/–	+/–			
N. flavescens	–	–	–	–	–			
M. catarrhalis	–	–	–	–	–			
K. denitrificans	+	–	–	–	–			

* – ONPG, *o*-nitrophenyl-β-galactosidase;

** – Glu-AMP, γ–glutamyl aminopeptidase;

*** – PIP, prolyliminopeptidase (hydroxyproline aminopeptidase; HPA);

**** – includes biovars subflava, flava, and perflava, which differ in their activity against saccharose and fructose;

\+ – change in medium colour (positive reaction);

– – absence of change in medium colour (negative reaction);

+/– – not consistent for the species.

for ONPG and usually from light-yellow to intense orange for Glu-AMP and PIP are considered positive. However, the sensitivity and specificity of these rapid enzyme-substrate tests have been shown suboptimal.

Results of sugar utilization and enzyme production tests for different Neisseria species and other oxidase-positive species such as *M. catarrhalis* and *Kingella denitrificans* are described in Table 1.

Immunological species confirmatory tests

Current commercially available immunological tests using monoclonal antibodies in direct immunofluorescence (DIF), co-agglutination and immune-enzyme tests are usually highly sensitive and specific for definitive species identification of *N. gonorrhoeae*. Furthermore, these assays are fast, require only a small amount of bacterial growth, and can also be applied to cultures obtained following primary inoculation. Consequently, isolation of pure cultures of gonococci is not necessary and gonococcal isolates may be identified 18–24 hours earlier than when sugar utilization or enzyme-substrate tests are used. However, the immunological tests are commonly more expensive.

Direct immunofluorescence

The test is most frequently based on using fluorescent-labelled monoclonal antibodies against the PorB protein, previously named as protein I or major outer membrane protein, of the gonococcal outer membrane. Strict adherence to the instructions of the manufacturer of each specific test is greatly important.

Direct immunofluorescence test procedure "MicroTrak":

- five typical colonies are taken with a bacteriological loop;
- the material is mixed in a drop of physiological saline solution or deionised water on a glass slide;
- air-dried and fixed in burner flame;
- 30 µl of fluorescent (fluoresceine isothiocyanate (FITC)) labelled monoclonal antibodies are applied;
- incubated for 15 minutes at 36 ± 1 °C in a moist chamber;
- rinsed with distilled water;
- air-dried;
- mounting fluid (buffered glycerine) is applied;
- overlaid with a cover-glass;
- examined using a fluorescence microscope at magnification ×1000. Positive results are recorded when typical diplococci showing apple-green fluorescence are identified.

The specificity and sensitivity of tests such as "MicroTrak" *N. gonorrhoeae* culture confirmation can be up to 99–100%. However, these vary depending on the test used, strict adherence to the instructions of the manufacturer, divergent clinical strains, and skills of the laboratory workers.

Coagglutination tests

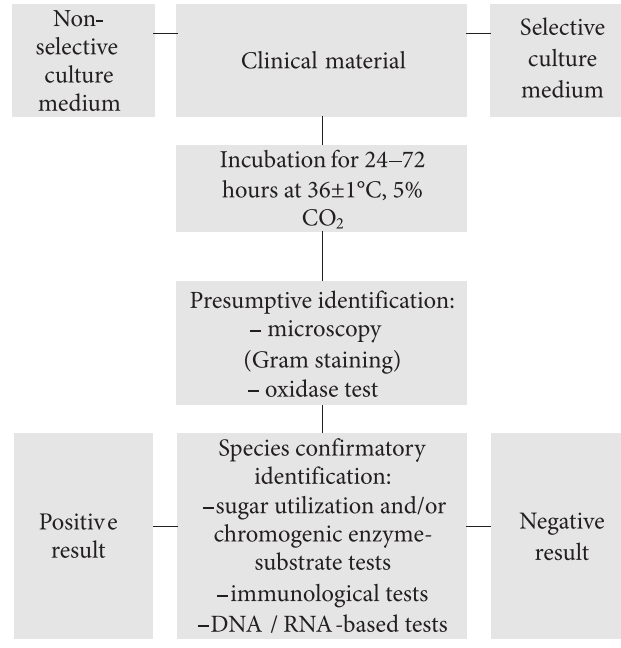
In the cell wall, *Staphylococcus aureus* (Cowan strain) contains a lot of surface-exposed protein A which can bind the Fc-portion of especially immunoglobulin G subclasses 2 and 4, leaving the Fab fragment free to react with an antigen. Heat-killed staphylococci covered with anti-gonococcal antibodies, i. e. the coagglutination reagent, are mixed with a boiled gonococcal suspension, resulting in flocculation (agglutination). Monoclonal antibodies against PorB located in the outer membrane of *N. gonorrhoeae* are used in highly sensitive and specific commercially available tests such as Phadebact GC Monoclonal Test, GonoGen I, and GonoGen II. In GonoGen II, monoclonal antibodies against PorB of gonococci are conjugated to colloidal gold instead of staphylococcal cells. Strict adherence to the instructions of the manufacturer of each specific test is greatly important.

Coagglutination test procedure (Phadebact):

- approximately ten pure colonies are suspended in 0.5 ml physiological saline solution;

- the suspension is boiled for 5–10 minutes;
- a drop of boiled bacterial suspension is applied on a slide;
- a drop of coagglutination reagent is added;
- the coagglutination reaction is assessed by gentle rocking of the slide for 1–2 minutes;
- the presence of agglutination is recorded as a positive reaction, while absence of agglutination is recorded as a negative reaction.

Attention!
For coagglutination tests, 18–24 hour cultures are required. It is hard to prepare a homogeneous suspension of colonies after 48 hours of incubation owing to, e. g., the release of nucleoprotein from autolysed microorganisms. It is important to strictly adhere to the instructions issued by the manufacturer of the specific test regarding procedure, utilized controls as well as interpretation of the results. Some *N. gonorrhoeae* strains may show a weak positive reaction, and it is highly important to distinguish it from a false-positive reaction due to, e. g., spontaneous agglutination.



Sensitivity and specificity of the coagglutination methods

The sensitivity and specificity of the current commercially available coagglutination methods can be up to 100%. However, these vary depending on the assays utilized, strict adherence to the instructions of the manufacturer, divergent clinical strains, age of the cultures, and skills of the laboratory workers. Rare gonococcal strains that do not react in the coagglutination assays and additional ones that give only a weak reaction have been described. Furthermore, false-positive reactions due to cross-reactions with other Neisseria species, e. g., *N. meningitidis*, *N. lactamica*, and *N. cinerea*, as well as spontaneous agglutination of the bacterial suspension have been recorded.

***N. gonorrhoeae* isolation and identification algorithm**

The following algorithm can be applied for identification of *N. gonorrhoeae* using culture and subsequent presumptive and definitive species confirmatory identification:

Quality control of *N. gonorrhoeae* culture

The quality of culture diagnosis of gonorrhoea is determined by a number of factors including:

- general qualifications of the physician and laboratory staff;
- accurate type of specimens;
- quality of sampling and the number of different sampling sites;
- adherence to recommendations regarding transportation of samples;
- composition and quality of culture media (see above);
- adherence to recommendations regarding inoculation and incubation conditions (see above);
- reagents, equipment and procedure used for species identification of the cultured bacteria (see above);
- interpretation of all the laboratory tests used (see above);
- availability of quality control of culture media (see below);
- availability of control strains and performance of quality control of reagents and tests used for culture and identification of different Neisseria species (see below).

Some problems that may arise during cultivation of gonococci and recommended solutions to these problems are shown in Table 2.

Table 2. ***N. gonorrhoeae* culture: possible problems and recommended solutions**

System parameter	Possible problems	Recommended solutions
Sampling of material, transportation and inoculation of culture media	• Incorrect type of specimen sampled; • incorrect sampling of material; • incorrect transport medium used; • incorrect transportation of sample; • incorrect inoculation of sample on the medium; • late inoculation	• Strict adherence to instructions regarding sampling; • strict adherence to instructions regarding transportation of samples; • strict adherence to instructions for inoculation
Selective culture medium	• Incorrect concentration of base agar or additional nutritive agents; • incorrect concentration of selective antibiotics; • toxicity of agar; • incorrect amount of medium per dish; • dehydration of agar; • culture medium used after expiry date	• Strict adherence to instructions for medium preparation and storage (see above); • examination of the growth ability of fastidious *N. gonorrhoeae* reference strains; • examination of the inhibitory capacity of the medium using reference strains, e. g. *Escherichia coli, Staphylococcus epidermidis, N. sicca, Candida albicans*

Table 2 continued

System parameter	Possible problems	Recommended solutions
Incubation	• Too low / high CO_2 concentration; • too low / high temperature; • humidity too low; • incubation time too short	• Strict adherence to instructions for incubation (see above); • control of CO_2 concentration; • control of temperature; • control of humidity
Evaluation of results	• Growth of colonies not characteristic of *N. gonorrhoeae*; • growth of accompanying saprophytic microflora; • incorrect interpretation of the oxidase reaction; • incorrect interpretation of the microscopic examination; • incorrect interpretation of the species confirmative assay(s)	• Strict adherence to instructions for preparation and quality control of the culture medium; • strict adherence to instructions for gonococcal identification (see above); • strict adherence to instructions for quality control of the reagents and assays used for gonococcal identification

Quality control of culture media

Before use, each new batch of medium should be controlled for its capacity to maintain gonococcal growth. This is performed by inoculation of one or several fastidious gonococcal reference strains or if reference strains are not available, inoculation of clinical material from a patient with confirmed gonorrhoea may be used. In addition, every new batch of medium should be examined regarding the inhibitory capacity of other bacterial species and fungi by inoculation of reference strains, e. g., *E. coli*, *S. epidermidis*, *N. sicca*, and *C. albicans*, as well as verified regarding sterility (by incubating a representative selection of the newly prepared plates at 36 ± 1 °C as well as at ambient temperature for 48 hours).

Quality control of Gram staining

Each new batch of chemical reagents for Gram staining should be quality-assured using Gram-positive and Gram-negative bacterial reference strains. In addition, every time the staining procedure is performed (or if a very low number of samples are analysed at the laboratory, at least once a week), the same reference strains should be included as controls.

Quality control of reagents for oxidase test

Each new batch of reagents should be quality-assured using oxidase-positive and negative reference strains.

- For positive control, e. g., *Pseudomonas aeruginosa* or *N. gonorrhoeae* strains can be used.
- For negative control, e. g., *S. aureus* or *E. coli* may be utilized.

Quality control of sugar utilization and chromogenic enzyme-substrate tests

Each new batch of in-house reagents or commercially available assays should be quality-assured using reference strains of appropriate bacterial species such as *N. gonorrhoeae, N. meningitidis, N. sicca, N. lactamica*, and *M. catarrhalis*. In addition, every time the species confirmative assay is performed, the same reference strains should be included as controls.

Quality control of immunological identification tests

Each new batch of in-house reagents or commercially available assays should be quality-assured using a *N. gonorrhoeae* reference strain (positive control) and a negative reference strain of other bacterial species. In addition, both these control strains should be included each time the method is employed.

Safety of personnel

This shall be ensured by compliance with the rules of work with possibly infected material in the specific country (use of gloves, laminated box, disinfectants, UV-radiation, etc.).

Laboratory conclusions based on results using culture

- *Neisseria gonorrhoeae* was isolated.
- *Neisseria gonorrhoeae* was not isolated.

NON-CULTURE METHODS FOR *N. GONORRHOEAE* DIAGNOSIS

For identification of *N. gonorrhoeae*, DNA / RNA-based methods (nucleic acid amplification tests (NAATs) such as polymerase chain reaction (PCR), strand displacement amplification (SDA) or transcription-mediated amplification (TMA), or hybridisation-based assays) may also be used. Many commercially available test systems enable identification of *N. gonorrhoeae* with a high level of sensitivity and, in most cases, high specificity. In addition, certified and comprehensively validated in-house assays may be utilized to reduce the costs. However, the results of DNA / RNA-based methods must be interpreted with care in the context of a clinical diagnosis, due to the fact that even after a successful treatment *N. gonorrhoeae* DNA may be detectable in specimens for up to 2–3 weeks. Culture is still the test recommended for symptomatic patients in order to provide a viable organism, especially for antimicrobial susceptibility testing. However, in some situations, NAATs appear to be more sensitive than culture, but this is highly dependent on the culture method used and the transportation of the specimen to the laboratory. In situations where transportation and laboratory facilities are optimal, culture is the overall method of choice. Conversely, when transportation takes time and culture conditions are not ideal, then NAATs are preferable. Consequently, the choice of a NAAT compared to culture is dependent on the facilities available for transportation and testing as well as on the population to be tested. In a high-risk population, culture of invasive specimens is still the test of choice. In a low-risk population and for

screening of risk groups, NAATs and non-invasive specimens may be more appropriate, i. e. urine in men or urine or low vaginal swabs in women. However, in a low-risk population, if the specificity of the used NAAT is not ideal, a high level of false-positives in comparison with true-positives will be identified. Overall, NAATs are the test of choice for non-invasive specimens but do vary in their sensitivity and specificity. The sensitivity of NAATs is usually highest for urines from men and lowest for urines from women. In women, in a low-risk population, the specimen of choice is self-taken vaginal swabs. Regarding screening of asymptomatic patients, NAATs can be used, but any positives should be confirmed using culture. Still, there are no licensed NAATs for rectal or pharyngeal specimens, mainly due to the fact that there is no evidence as yet that any of the NAATs are reliable, especially comprising adequate specificity, for rectal or pharyngeal sites. The DNA / RNA-based methods can be used also for species confirmation of *N. gonorrhoeae* from primary cultures. For this purpose, the material that has been sampled by a loop from a suspicious colony can be applied in a sterile Eppendorf test tube, or equivalent tube, with 100 µl of physiological saline solution and, subsequently, DNA can be extracted and analysed. However, this test does not exclude the necessity of presumptive *N. gonorrhoeae* identification, as described above. If clinical specimens are to be analysed, specimens are treated and DNA is extracted as well as analysed in compliance with the instructions of the manufacturer of the specific diagnostic test.

Advantages of DNA / RNA-based methods for *N. gonorrhoeae* diagnostics:

- high sensitivity (in most cases);
- high specificity (in most cases, but exceptions exist);
- rapid with possible automation;
- possible use of non-invasive specimens (e. g., urine or vaginal swabs);
- possible use of self-taken samples (e. g., urine or vaginal swabs);
- absence of necessity for viable bacteria;
- in some cases, possibility of using pooled samples;
- possible detection of several agents (e. g., *Chlamydia trachomatis* and *N. gonorrhoeae*).

Disadvantages of DNA / RNA-based methods for *N. gonorrhoeae* diagnostics:

- problems with specificity in some test systems (e. g., false-positive results due to presence of non-pathogenic Neisseria species in a sample);
- problems with sensitivity in some test systems (e. g., presence of inhibitors and/or many gonococcal strains are lacking the selected genetic target, e. g. *cppB* gene);
- expensive equipment and reagents;
- risk of specimen and amplified product cross-contamination;
- absence of effective protocols for some types of clinical specimens (e. g., rectal or pharyngeal);
- do not allow antimicrobial sensitivity testing or other phenotypic characterisation of *N. gonorrhoeae*;
- may detect non-viable bacteria even after a successful treatment and therefore may be suboptimal for test of cure, at least 1–2 weeks posttreatment.

Attention!
DNA / RNA-based methods are mainly not recommended as the sole diagnostic method (see above).

Brief characteristics of some commercially available non-culture diagnostic methods based on detection of nucleic acid of *N. gonorrhoeae* are given in Table 3.

These methods are intended for detection of *N. gonorrhoeae* in urogenital specimens. Especially the NAATs show, in most cases, both a high sensitivity and specificity. These facts enable their use in screening applications when testing clinical urogenital samples, including those obtained by non-invasive methods (e. g., urine). However, inhibitory substances of the samples and the fact that some *N. gonorrhoeae* strains may lack specific sequences targeted in some of the NAATs can lower the sensitivity. In addition, the specificity of several of the DNA / RNA-based methods has been shown suboptimal and many false-positive samples due to presence of, e. g., commensal Neisseria species such as *N. cinerea*, *N. lactamica* and *N. subflava* have been identified. Nevertheless, novel in-house PCRs, which target the *porA* pseudogene of *N. gonorrhoeae*, have so far proved to have a satisfactory specificity and seem very promising. This *porA* gene / pseudogene is absent in commensal Neisseria species, and the *porA* gene of *N. meningitidis* is sufficiently divergent to be discriminatory between the two human pathogenic Neisseria. Any in-house amplification test should have equal sensitivity and specificity to commercial assays. Culture and subsequent confirmative species identification should be carried out for all extragenital specimens, if antimicrobial sensitivities are required, and for specimens with medico-legal consequences such as child or sexual abuse.

Table 3. **Commercially available non-culture diagnostic methods based on detection of *N. gonorrhoeae* nucleic acids**

Genetic target	Method
16S rRNA gene	Probe hybridisation
Chromosomal and cryptic plasmid sequences	Probe hybridisation
cytosine DNA methyltransferase gene	PCR
16S rRNA gene	NASBA
16S rRNA gene	TMA
pivNG gene	SDA

Procedures

DNA extraction and performance of assay

This is carried out in strict adherence to the instructions of the manufacturer of the specific commercial diagnostic test or in accordance with the written guidelines of the in-house assay. Written guidelines regarding the cleaning of the equipment and the utilized laboratory facilities should also exist. For example, after finishing the work at each working area the bench should be thoroughly cleaned using DNA / RNA degrading solution in order to avoid the spread of previously amplified nucleic acid.

Interpretation of results

There is no doubt that the development and subsequent application of NAATs represent a major advance in the diagnosis of many infectious diseases. However, particular care should be taken in the choice of molecular diagnostic tests for gonorrhoea and their subsequent interpretation, even when the tests appear to have good performance characteristics. This is particularly important where screening is applied to low prevalence populations. At a prevalence of disease of 50%, a test with both a sensitivity and specificity of 95% will correctly identify infected individuals and uninfected individuals in 95% of cases, but at a disease prevalence of 5% the test has a positive predictive value (the percentage of those persons with a positive test result who are actually infected) of only 50%, but a negative predictive value (the percentage of those persons with a negative test who are truly uninfected) of 99.7% (Table 4).

Table 4. **Positive and negative predictive values of a diagnostic test at varying disease prevalence when sensitivity and specificity are 95%**

Prevalence of disease (%)	Positive predictive value (%)	Negative predictive value (%)
1	16.1	99.9
2	27.9	99.9
5	50.0	99.7
10	67.9	99.4
20	82.6	98.7
50	95.0	95.0
75	98.3	83.7
100	100.0	–

As a further example, at a gonorrhoea prevalence of 2%, a test that has a sensitivity of 90% and a specificity of 98% has a positive predictive value of only 47.9% (Table 5).

It should be noted that even if both the sensitivity and specificity of the test were 99%, the positive predictive value would be only 66.9% at that prevalence of disease.

These figures clearly indicate that care should be taken when disclosing positive results to persons at a low risk of infection in low prevalence settings, since the high rate of false-positive results may have unfortunate implications for partner notification.

Quality control

In each DNA extraction and subsequent analysis of samples, an internal positive control, which allows detection of substances in samples that inhibit the amplification reaction and controls the quality of sample preparation, and a negative control such as sterile, distilled water should be included.

Certified and registered reference panels comprising coded control specimens should ideally be used for intra- and inter-laboratory quality control for DNA / RNA-based diagnostics (external control). The use of specimen panels is standard for test system operation. These act as indicators of sensitivity, specificity and reproducibility independently of the test systems used.

Comprehensive evaluation and validation of the assay utilized, strict adherence to the instructions of the manufacturer or written guidelines for in-house assays, and quality assurance, i. e. internal and external controls, are crucial for a highly sensitive and specific diagnosis of *N. gonorrhoeae* using DNA / RNA-based methods.

Safety of personnel

All specimens should be treated and handled as potentially containing infectious agents, therefore:

- do not pipette with mouth;
- it is forbidden to smoke, eat or drink at the workplace;
- use disposable gloves and protective clothes;
- thoroughly wash your hands after finishing work;
- all materials, including the used devices, reagents and specimens, should be autoclaved or soaked in a 1% solution of chlorine.

Attention!

Ethidium bromide is a strong mutagen! Consequently, during all work with ethidium bromide staining and stained agarose gels, use gloves.

Laboratory conclusions based on results using DNA / RNA-based methods

- Nucleic acid specific for *Neisseria gonorrhoeae* was detected.
- Nucleic acid specific for *Neisseria gonorrhoeae* was not detected.

SEROLOGY

During past decades, many tests for detection of antibodies against *N. gonorrhoeae* in serum, such as complement fixation, latex agglutination, immunofluorescence, and immunoblotting have been developed. However, none of these is able to distinguish a current, active infection from one that has previously been treated. In addition, in most cases these methods show a too low sensitivity for diagnosis of uncomplicated gonorrhoea. Serological tests should not be routinely used for diagnosis of gonorrhoea.

Table 5. **Predictive value of a positive diagnostic test over a range of sensitivities and specificities when the actual prevalence of disease is 2%**

Specificity (%)	Sensitivity (%)							
	50%	60%	70%	80%	90%	95%	98%	99%
50	2.0	2.4	2.8	3.2	3.5	3.7	3.8	3.9
60	2.5	3.0	3.4	3.9	4.4	4.6	4.8	4.8
70	3.3	3.9	4.5	5.2	5.8	6.1	6.2	6.3
80	4.8	5.8	6.7	7.6	8.4	8.8	9.1	9.2
90	9.2	10.9	12.5	14.0	15.5	16.2	16.7	16.8
95	17.0	19.7	22.2	24.6	26.9	27.9	28.6	28.8
98	33.8	38.0	41.7	44.9	47.9	49.2	50.0	50.2
99	50.5	55.0	58.8	62.0	64.7	66.0	66.7	66.9

DETERMINATION OF *N. GONORRHOEAE* ANTIBIOTIC SENSITIVITY AND RESISTANCE

General

Over the recent decades, intermediate sensitivity and resistance to most traditional antibiotics used in the treatment of gonorrhoea have emerged and increased worldwide. This resistance occurs as both chromosomally mediated and plasmid-mediated (penicillins and tetracyclines). However, there are marked geographical variations regarding the levels of resistance, and antibiotic therapy should ideally be confirmed by local, national and international surveillance of sensitivity. Accordingly, it is often necessary to determine the antibiotic sensitivity of gonococcal isolates. The determination of antibiotic sensitivity can be carried out in all laboratories where gonococcal culture can be performed. However, determination of the sensitivity of all *N. gonorrhoeae* isolates to antibiotics may not be needed for routine use in all smaller laboratories.

Definitive indications for application of antibiotic sensitivity testing:

- epidemiological testing regularly conducted by, e. g., reference laboratories to obtain local, regional or national information regarding antibiotic resistance of gonococci;
- failure of gonorrhoea treatment using recommended standard antimicrobial treatment;
- monitoring of clinical efficacy of recommended standard strategies of gonorrhoea treatment;
- study of anti-gonococcal activity of new antimicrobial agents.

Plasmid-mediated antibiotic resistance of *N. gonorrhoeae*

During recent decades, an increasing number of *N. gonorrhoeae* isolates have emerged that produce the enzyme β-lactamase (TEM-1; penicillinase) which inactivates benzylpenicillin (penicillin G), ampicillin, amoxicillin, etc. Consequently, this results in failure of gonorrhoea therapy with many penicillins. In addition, many / most gonococcal strains are nowadays resistant to tetracyclines. High resistance also to these antibiotics is due to acquisition of specific plasmids (*tet* (M)-carrying conjugative plasmids).

For epidemiological purposes, gonococcal strains can be tested for plasmid-mediated resistance to penicillins and tetracyclines.

β-lactamase activity of gonococci

The β-lactamase activity of gonococci can be determined by means of the chromogenic cephalosporin test which is highly reliable and convenient. Discs saturated with cephalosporin nitrocefin are used in the test. The test is based on the principle that β-lactamase hydrolyses the β-lactam ring of nitrocefin, and this results in a colour change from yellow to pink or red, which is visible with the naked eye.

***Test procedure*:**

- a nitrocefin disc is placed in a clean, dry and empty plate (Petri dish) or on a glass slide and is moistened with distilled water. Alternatively, the disc can be placed in a tube containing 0.3 ml sterile distilled water;
- the material from 5–10 gonococcal colonies is placed on the disc with a bacteriological loop, alternatively, suspended in the tube;
- change of colour is observed for 5 seconds – 15 minutes or, if the tube test is performed, after 10–30 minutes of incubation at room temperature or at 36 ± 1 °C;
- should the colour change from yellow to pink or red within 5 seconds – 15 minutes (usually within 1 minute), the test is considered as positive. Absence of colour change after 15 minutes is considered as a negative test. Alternatively, if the tube test is performed, the results are evaluated after 10–30 minutes of incubation.

A β-lactamase-positive reference strain of, e. g., *Haemophilus influenzae* or *N. gonorrhoeae* is used as a positive control, while a β-lactamase-negative reference strain of, e. g., *Enterococcus faecalis* or *N. gonorrhoeae* acts as a negative control.

It is possible to use also other tests (acidometric, iodometric), though they are more technically complex.

Attention!

To perform the β-lactamase test, it is required to use a gonococcal culture that has not been incubated for more than 24 hours. If commercially available discs are utilized, a strict adherence to the instructions of the manufacturer of the specific test is crucial. A positive nitrocefin test indicates that the strain is resistant to penicillin, ampicillin, amoxicillin, etc.

Plasmid-mediated resistance to tetracyclines

Plasmid-mediated resistance of gonococci to tetracyclines can be determined using a disc containing 30 μg tetracycline by the disc diffusion method (see below). A zone of growth inhibition less than 19 mm in diameter usually indicates plasmid-mediated resistance to tetracyclines.

Methods for antibiotic sensitivity testing

The sensitivity of *N. gonorrhoeae* to antibacterial agents can be determined by several methods, e. g., disc diffusion, agar dilution, and the Etest method. Various types of test medium, including the non-selective culture medium used for gonococcal identification, can be used.

The disc diffusion method is more qualitative for detection of gonococcal resistance to antibiotics.

The quantitative tests include agar dilution and the Etest method that allow measurement of the minimum inhibitory concentration (MIC) of an antibiotic, i. e. the lowest antibiotic concentration that can inhibit the visible growth of the microorganism. Ideally, one of these methods should be used for antibiotic sensitivity testing of *N. gonorrhoeae*. However, these methods are usually more expensive.

Antimicrobial agents for determination of sensitivity / resistance of *N. gonorrhoeae*:

- the penicillin group: benzylpenicillin, ampicillin;
- the cephalosporin group: ceftriaxone, cefotaxime, cefixime;
- the tetracycline group: tetracycline, doxycycline;
- the macrolide group: erythromycin, azithromycin;
- the fluoroquinolone group: ciprofloxacin, ofloxacin;
- the aminoglycoside group: kanamycin, gentamycin;
- the aminocyclitol group: spectinomycin.

The **mandatory minimal** set of antibacterial drugs for the antibiotic sensitivity testing of gonococci should include representatives of various antibiotic groups routinely used for the treatment of gonorrhoea, namely: ceftriaxone / cefixime, ciprofloxacin / ofloxacin, and spectinomycin. Optionally and for epidemiological purposes at reference laboratories, e. g., benzylpenicillin / ampicillin, cefotaxime, tetracycline / doxycycline, azithromycin / erythromycin, and kanamycin / gentamycin may in addition be analysed.

In different geographic areas, the choice of antibiotics included in the testing may differ and the selection of antibiotics should be based on the routine therapy applied locally. Information on the test results should be conveyed to the physicians managing the cases.

Disc diffusion method

General

The disc diffusion method can be performed in any laboratory that is performing culture of gonococci. This method is based on the capacity of antibiotics to diffuse from paper discs into the culture medium, inhibiting the growth of gonococci inoculated on the surface of the medium. Only highly standardized and qualitatively assured medium, discs and procedures, including interpretation of results, should be used for determination of the antibiotic sensitivity of gonococcal isolates.

Commercially available discs of standard size and appropriate antibiotic concentration should be used in the disc diffusion method. Unused antibiotic discs should be stored at 4 ± 2 °C until the expiry date, with exception of discs containing β-lactam antibiotics, which should be stored frozen at –20 °C. Disc packages should be allowed to assume ambient temperature prior to use.

Procedure for disc diffusion method

A standard dilution of gonococci, which corresponds to a 0.5 McFarland standard and contains approximately 1.0×10^8 CFU/ml, is applied on preferably 90–100 mm plates containing 25 ml of medium in order to obtain a homogeneous, semiconfluent growth of gonococci after incubation. The diluted suspension of gonococci must be used within 15 minutes following its preparation.

- Gonococcal suspensions may be applied to the agar surface by one of the two methods:
  - inoculate a plate containing no antibiotic (negative control);
  - Application of suspension using sterile cotton swabs. For this procedure, it is recommended to use cotton swabs on wooden sticks. Swabs made from synthetic materials do not absorb the suspension in quantities sufficient for inoculation over the entire agar surface. A swab is immersed in the standard suspension, and then the excess is removed by pressing the swab against the wall of the test tube. The suspension is applied by stroking movements in three directions turning the agar plate by 60°. The agar surface may need to be treated with the swab up to 3–4 times.
  - Application of suspension using a pipette. Sufficient volume of suspension to cover the entire agar surface of the culture medium, i. e., 3–4 ml for a 90–100 mm plate, is dispensed with a pipette and evenly distributed over the agar surface by gentle rocking. Excess inoculum is removed with a sterile pipette. The plates are then predried at ambient temperature for 5–10 minutes.
- In 5–10 minutes, antibiotic discs are applied to the agar surface by means of sterile forceps or an automatic dispenser. The distance between discs and between discs and the edge of the agar surface should be at least 15–20 mm. Therefore, no more than 6 discs should be placed on a 90–100 mm plate. No discs should be placed in the centre of the plate. Discs should be applied uniformly in contact with the agar surface. For this purpose they should be carefully pressed with the forceps. After application of a disc on the surface of the culture medium, this should not be moved to a new place.
- Immediately after the discs have been applied to the agar surface, the plate should be placed in an incubator containing 5 ± 2% CO_2, approximately 70% humidity, at a temperature of 36 ± 1 °C for 20–24 hours (candle extinction jar and ordinary incubator at 37 °C are also applicable). Increasing the time between disc application and the start of incubation can distort the results. Results are recorded by measuring the diameter of the area (zone) of complete inhibition of visible gonococcal growth. Very small single colonies detected within the growth inhibition area should be neglected.

Attention!

18–24 hour cultures of gonococci are required for antimicrobial susceptibility testing.

Agar dilution method

General

The method is based on the preparation of a series of successive dilutions of antibiotics in culture medium and the subsequent inoculation of examined gonococcal isolates on the plates containing the dilutions. The degree of strain sensitivity is judged according to inhibition of bacterial growth at a certain concentration of the antimicrobial agent.

For antibiotic sensitivity testing of *N. gonorrhoeae*, the agar dilution method is considered as the reference method. The method determines the minimum inhibitory concentration (MIC) of antibiotics. This method is mostly utilized in reference laboratories to obtain information regarding antibiotic resistance of gonococci and to monitor the local, regional and national variations. For routine use, a less labourious method, i. e. preferably Etest but also a highly evaluated, standardized and quality assured disc diffusion can be used.

Pre-prepared media containing various concentrations of antibiotics should not be stored in the refrigerator for over a week because of a possible loss of antimicrobial activity.

Procedure for agar dilution method

A standard dilution of gonococci, corresponding to a 0.5 McFarland standard containing approximately 1.0×10^8 CFU/ml, is used in the agar dilution method. After the suspension is prepared, it should be inoculated within 15 minutes on agar plates containing various antibiotic concentrations. Several gonococcal strains may be placed on a single plate by means of a special replicator.

- The inoculation of plates should be performed in the following order:

- inoculate a plate containing no antibiotic (negative control);
- inoculate antibiotic plates in increasing concentrations of the antibiotic;
- finally, inoculate one more plate containing no antibiotic (second negative control) to exclude contamination, which could have occurred during the process of inoculation;
- *N. gonorrhoeae* reference strains of known sensitivity / resistance to tested antibiotics should be included in every run.

• Immediately after inoculation, the plates are placed in an incubator containing 5 ± 2% CO_2, approximately 70% humidity, at a temperature of 36 ± 1 °C for 24 hours (candle extinction jar and ordinary incubator at 37 °C are also applicable).

• Plates are inspected for growth after 24 hours. If there is some doubt regarding sufficient growth of *N. gonorrhoeae* in the inoculation locations, incubation may be extended by another 24 hours.

• Results are documented after 24 or 48 hours. The lowest antibiotic concentration, at which gonococcal growth is absent is considered the minimum inhibitory concentration for that antibiotic.

Etest method

General

The Etest method is based on the ability of pre-prepared concentration gradients of antibiotics to diffuse from graduated paper strips into the culture medium and, consequently, inhibit the growth of gonococci inoculated on the agar surface. Inhibition of growth occurs in a tear-shaped area, which intersects the strip at the level of the MIC.

Procedure for Etest method

• A standard dilution of gonococci, which corresponds to a 0.5 McFarland standard and contains approximately 1.0×10^8 CFU/ml, is applied on 90–100 mm plates (25 ml medium) or 140–150 mm plates (60 ml medium) in order to obtain a homogeneous, semiconfluent growth of gonococci after incubation. The suspension of gonococci must be used within 15 minutes following its preparation;

• inoculation of the gonococcal culture on the plates is carried out in a manner similar to the procedure described above for the disc diffusion method;

• Etest strips containing concentration gradients of different antibiotics are applied to the agar surface using sterile forceps. Strips should be applied uniformly in contact with the agar surface. For this purpose, they should be carefully stroken from a low to high concentration with the forceps. After application of a strip, the latter should not be moved to a new place. One strip may be applied on each 90–100 mm plate. However, up to 4 strips may be used on plates with a diameter of 140–150 mm, if they are located radially;

• immediately after inoculation, the plates are placed in an incubator containing 5 ± 2% CO_2, approximately 70% humidity, at a temperature of 36 ± 1 °C for 16–18 hours (candle extinction jar and ordinary incubator at 37 °C are also applicable).

Most important

Antibiotic sensitivity testing is greatly affected by the methodological procedure, i. e., the size of inoculum from fresh culture, the content and volume of culture medium, the quality of antibiotics / discs / Etest strips, incubation conditions, and determination of inhibition. Consequently, a high level of optimisation, standardisation and quality assurance of the antibiotic sensitivity testing method is crucial. The interpretative criteria (susceptible (S), intermediate (I) and resistant (R) breakpoints) that are used need to be optimised for each specific method. Furthermore, on a regular basis (dependent on the number of samples), *N. gonorrhoeae* reference strains with known sensitivity / resistance to the different tested antibiotics should be analysed in all methods.

PRESERVATION OF *N. GONORRHOEAE* STRAINS

A viable *N. gonorrhoeae* strain can be maintained by continuous passage every day on, e. g., chocolate agar.

For a short period of time (up to 3 months), if placed in test tubes containing 0.5 ml of trypticase-soyabean broth with 20% glycerol, a fresh culture can be preserved in the freezer at –20 °C. Extended storage at this temperature is not desirable as gonococci may lose their viability.

For extended storage (for many months or years) cultures in an appropriate preservation medium should be placed at –70–80 °C. Gonococcal strains can also be preserved by lyophilisation or freezing in liquid nitrogen.

QUALITY ASSURANCE CONTROL SYSTEM

A permanent, ongoing and implemented quality assurance control programme should be an integral part of every microbiological laboratory. The following factors may affect the quality of laboratory work involved in gonorrhoea diagnostics: the quality and composition of transportation media, culture media, incubation conditions, reagents, equipment, and laboratory personnel.

All laboratories should have common, clearly described and written as well as implemented guidelines for all procedures involved in the work with clinical material. Testing of individual samples by the same methods should always yield comparable results.

The international standards of quality control "Medical laboratories – Special requirements to quality and competence" (ISO 15189, 2003) are highly suitable for guidance for all laboratories engaged in STD diagnostics.

ACKNOWLEDGEMENTS

The present work was supported by a grant from the East Europe Committee of the Swedish Health Care Community, SIDA, Sweden.

Received 18 December 2006
Accepted 16 May 2007