Serological Diagnosis of Gonorrhoea Using Gonococcal Pili as Antigen

PROEFSCHRIFT

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Arnoldus Pieter Oranje
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Promotoren : Prof. Dr. M. F. Michel
Prof. Dr. E. Stolz

Co-referenten: Prof. Dr. J. Huisman
Prof. Dr. H. C. Zanen

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"A Rolling Stone
gathers no moss"

List of Abbreviations

A	=	absorbance
CV	=	coefficient of variation
ELISA	=	enzyme-linked immunosorbent assay
GCFT	=	gonococcal complement fixation test
GC	=	gonococcal infections, gonorrhoea
IgA	=	immunoglobulin A
IgG	=	immunoglobulin G
IgM	=	immunoglobulin M
IHA	=	indirect haemagglutination reaction
n	=	number of patients or number of examinations
NGU	=	non gonococcal urethritis
OPD	=	orthophenylene diamine
PBS	=	phosphate-buffered saline
PV	=	predictive value
RIA	=	radioimmunoassay
SE	=	standard error
STD	=	sexually transmitted diseases

List of Definitions*

D1 = date of first examination
D2 = generally 11-22 days after D1 (otherwise indicated)

$$\text{Sensitivity} = \frac{\text{diseased persons with positive test}}{\text{all diseased persons tested}} \times 100\%$$

$$\text{Specificity} = \frac{\text{non-diseased persons with negative test}}{\text{all non-diseased persons tested}} \times 100\%$$

Predictive value of positive test = PV (+)

$$\text{PV}(+) = \frac{\text{number of diseased persons with positive test}}{\text{total number of persons with positive test}}$$

Predictive value of negative test = PV (−)

$$\text{PV}(-) = \frac{\text{number of non-diseased persons with negative test}}{\text{total number of persons with negative test}}$$

ns = not significant, $p > 0.10$
$p < 0.05$ significant
$0.05 < p < 0.10$ nearly significant

$$\text{Coefficient of variation (CV)} = \frac{\text{standard error}}{\text{mean}} \times 100\%$$

* adopted from
Hart G.
Screening to control infectious diseases: Evaluation of Control Programs for Gonorrhea and Syphilis.
Reviews of Infectious diseases, 1980,2: 701-712

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CHAPTER 1

General Introduction

Introduction

Gonorrhoea is an infectious disease caused by *Neisseria gonorrhoeae.* Its uncomplicated form can be found in the urogenital tract, rectum and oropharynx in both males and females. The disease is one of the sexually transmitted diseases, and as a rule is transmitted via sexual intercourse. At this time, gonorrhoea is among the most notifiable infectious diseases in the world.

Neisseria gonorrhoea

In 1879 Neisser discovered the gonococcus (*Neisseria gonorrhoeae*) and identified it as the probable pathogen of gonorrhoea[1]. In 1885 Bumm succeeded in inducing gonorrhoea by inoculating pure cultured gonococci into a male urethra.[1]

The bacterium is a species of the genus *Neisseria* of the family *Neisseriaceae.* The *Neisseriae* are non-motile, non-sporing, Gram-negative diplococci. The nutrient media used to culture gonococci must meet very specific requirements. They autolyse rapidly and their resistance to changes in physical and chemical conditions is low.

Gonococci can be distinguished from other *Neisseria* species by sugar fermentation tests and immunofluorescence techniques[2]. Gonococci ferment only glucose, but not maltose, lactose and saccharose. Immunofluorescence techniques make use of specific antisera. On an agar medium, gonococcal strains can assume various colony types, the most important of which are T1, T2, T3 and T4 (Kellogg et al.)[3]. Cultivation of freshly isolated clinical material generally produces the small colony types T1 and T2. After non-selective cultivation these types change into T3 and T4. Whereas gonococci of colony types T1 and T2 cause gonorrhoea after intra-urethral inoculation of human volunteers, this cannot be achieved with T3 and T4 gonococci.

Jephcott et al. (1971)[4] and Swanson et al. (1971)[5] independently demonstrated pili on type 1 and 2 gonococci. These pili prove to play an important role in the attachment of gonococci to human cells[6]. The cell envelope of the gonococcus (figure 1) consists of a cytoplasmic membrane, a peptidoglycan layer and an outer membrane. There are indications that the gonococcus forms a capsule of polysaccharides outside the outer membrane [7,8,9].

Epidemiological investigation requires methods to establish the identity of a particular gonococcal strain. Catlin (1973)[10] described a typing system (auxotyping) which makes it possible to divide strains into groups on the basis of growth requirements. Johnston et al. (1976)[11] and Sandström and Danielsson

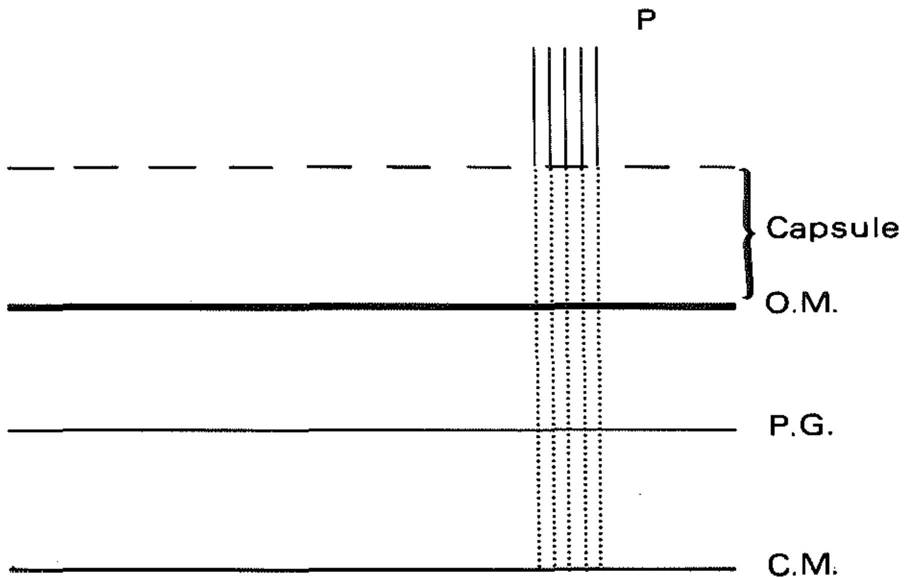

Fig. 1. Composition of the gonococcal cell wall.

P = Pili
O.M. = Outer membrane
P.G. = Peptido glycan layer
C.M. = Cytoplasmic membrane

(1980)[12] described typing systems in which the composition of the outer membrane and of other surface proteins is regarded as characteristics of a strain.

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CHAPTER 2

Serological Tests for Detection of Gonorrhoea

Although in the course of time many tests have been designed, we are still in need of a sensitive, specific serological test to detect all forms of gonorrhoea. In The Netherlands, the GCFT is as a rule used to detect gonorrhoea serologically. It can be stated in general that the serological tests so far developed are unsuitable for detection of (usually recent) anterior urethritis in the male. The available tests are more suitable for detection of asymptomatic infections in females, who are an important source of new infections. Serological tests are of great importance especially for gonorrhoeal infections associated with complications, because in these infections the gonococcus involved can often not be cultured, while serological tests show a relatively high sensitivity.

Whole cells, crude preparations and extracts were initially used as antigen in various serological tests, without much success or emulation (Table 1)[1-23]. In recent years, attempts have been made to enhance the sensitivity and specificity of tests for detection of gonorrhoea, especially by using purified antigens (pili, outer membrane protein antigens) and by introducing advanced techniques such as RIA and ELISA (Table 2)[24-32].

Crude antigen

The GCFT using a crude antigen is the serological test so far most widely used in the diagnosis of gonorrhoea. The test was introduced in 1906 by Müller and Oppenheim, who prepared an unpurified antigen from a single strain; Teague and Torrey (1907) subsequently used antigen prepared from several strains[4]. This reaction is still being performed routinely in The Netherlands. The antigen used is a crude extract prepared from a single strain (Oliver). The test is inadequate due to low specificity but above all due to lack of sensitivity. If interpreted with care, this test can nevertheless be used as an indicator in the diagnosis of complicated gonorrhoeal infections.

Gonococcal pili

Buchanan et al. (1973)[24] described a RIA in which gonococcal pili are used as antigen and positive results are obtained in 85% of females with uncomplicated gonorrhoea; its specificity, moreover, proved to be very high. Subsequent investigators confirmed the antigenic properties of gonococcal pili in RIA, IHA and ELISA systems, but scored lower than Buchanan et al. (Table 2). A disadvantage of these tests is that antibodies to pili as a rule prove to be of the IgG class, and consequently persist longer.

Table 1 *Serological tests for detection of gonorrhoea, using crude antigens*

Principal antigens used	Test	Sensitivity %			Specificity % (control groups)
		Females with uncompl.# GC	Males with uncompl. GC	Patients with compl. GC	
Crude antigen suspension; polyvalent/monovalent preparations	GCFT[1-7, 29]	8-88	13-50	44-95	89- 99 (blood donors)
Alkaline extract sonicate; LPS*	haemagglutination[8-10]	49-84	23-77	–	80- 98 (nuns, children, pregnant women)
Precipitins (LPS)	precipitation[11-12]	60	73-88	–	68-100 (pregnant women, females, laboratory personnel)
Crude sonicate fractions	flocculation (incl. micro)[6, 13-15]	51-86	50-80	–	85- 96 (blood donors, nuns, monks).
Whole gonococci	IFA[6, 16-20]**	61-79	20-56	86	89- 98 (nuns, low-risk groups)
Commercial antigen	Gonosticon dry dot[21, 22]	61	11	–	75- 98 (low-risk groups)
Soluble antigen	RIA[23]	100	93	–	60- 80 (females, military personnel)

* LPS = lipopolysaccharide.
** IFA = indirect fluorescent antibody test.
\# (un)compl. = (un)complicated.

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CHAPTER 3

Aim and Design of the Study

The studies described in the following chapters were performed in an attempt to contribute to a solution of the problems mentioned in chapter two. For the purpose of these studies, material had to be collected from patients and several techniques had to be developed. Material and techniques were then used to study a number of specific problems concerning the serological diagnosis of gonorrhoea.

1. Almost the entire material was collected from patients attending the STD clinic of the Rotterdam University Hospital Dijkzigt. Gonococci were isolated from all patients with gonorrhoea, and stored. Sera were collected from patients with and without gonorrhoea. The patients were divided into well-defined groups on the basis of history, sex and diagnosis. Blood samples were obtained on the day of the first examination (D1) and on at least one other day (D2), generally 11-22 days after D1. More than two sera were collected from some patients.

2. For serodiagnostic purposes an ELISA was developed, using a purified gonococcal pilus preparation from a single strain as antigen. The sera were also submitted to two tests routinely used elsewhere: an IHA using a pilus antigen from another gonococcal strain (used at the Statens Seruminstitut, Denmark), and a GCFT using a crude extract from a single gonococcal strain as antigen, (used at the National Health Institute, Bilthoven, The Netherlands).

3. The first objective of this study was to evaluate the sensitivity and specificity of the abovementioned ELISA as applied to sera from various groups of patients. After application of the IHA and the GCFT to the same sera, the results were compared with those of the ELISA.

4. Next, the influence of the choice of gonococcal pilus antigen on the sensitivity and specificity of the serological test was studied.

5. Finally, a study was made of the contribution of the ELISA to the diagnosis of gonorrhoea when this test was applied to females without complaints who attended the STD clinic for examination.

CHAPTER 4

Material and Methods

Isolation and Purification of Gonococcal pili

Pili used in this study were isolated by two methods. In both cases we started with highly piliated gonococci of colony type 1 or 2. In the procedure described by Robertson et al.[1], gonococci are homogenized and, after differential centrifugation, purified over a caesium chloride gradient. In the procedure described by Hermodson et al.[2] the gonococci are similarly processed but purified by precipitation with ammonium sulphate. Detailed descriptions of the two methods follow.

Method of Robertson et al.[1] (modified by Oranje)

Gonococci were cultured on Difco GC medium or Kellogg solid nutrient medium during 20 hours at 37°C in humid air containing 5% carbon dioxide. Colony types 1 and 2 were selectively transferred in further inoculations and stored at –70°C in aliquots filled with liquid Difco GC medium (without agar) to which 10% glycerol had been added. For the production of pili, gonococci from such an aliquot were cultured overnight and then checked for colony type. In the case of doubt the presence of pili was established electron-microscopically. The gonococci were then suspended in normal saline and the suspension was dripped onto plates (with a diameter of 9 cm) and spread with the spatula (0.1 ml/plate = 10^9 gonococci/ml). Type 1 or 2 gonococci were harvested from 200 Difco GC medium or Kellogg solid nutrient medium incubated during 20 hours at 37°C in humid air containing 5% CO_2 (in a CO_2 incubator type T 303, Assab, Sweden) and suspended in ice-cold (0°C) 0.01 M Tris HCl buffer pH 8.0. The harvest from 50-80 plates was suspended in 15 ml buffer. For release of the pili the gonococci were homogenized during 2 minutes in an MSE homogenizer. Gram staining was used to check the suspensions for possible contamination by other bacteria. To disaggregate the pili, 5.1 g sucrose (1M) was added to 15 ml suspension. This mixture was briefly shaken on the vortex mixer, stored during 30 minutes at 0°C, and homogenized during 15 seconds. The pili were then separated from the bacteria by centrifuging them twice at 10,000 x g and twice at 15,000 x g. The two supernatants were combined and NaCl was added to a concentration of 1 M. The impure pili were sedimentated by centrifuging at 100,000 x g during 4 hours at 4°C. The sediment was resuspended in 0.01 M Tris HCl buffer pH 8.0, and caesium chloride was added to a concentration of 40% (w/v). The pili were then separated and treated with a Teflon homogenizer. Gradient centrifugation (100,000 x g) took place in a swing-out rotor during 48 hours at 4°C. A band

consisting of purified pili was obtained at a density of 1.30. Ultimately the concentrated pili were drained off by suction, centrifuged at 100,000 x g during 3 x 1 hour at 4°C, and washed in PBS pH 7.8 to which 0.38 M NaCl had been added. The pili were stored in PBS pH 7.8 (0.02% w/v NaN_3). The quality of the pilus preparation was visually checked electron-microscopically. If it was insufficiently pure it was rejected, or caesium chloride gradient centrifugation was repeated.

Method of Hermodson et al.[2] (modified by Reimann)

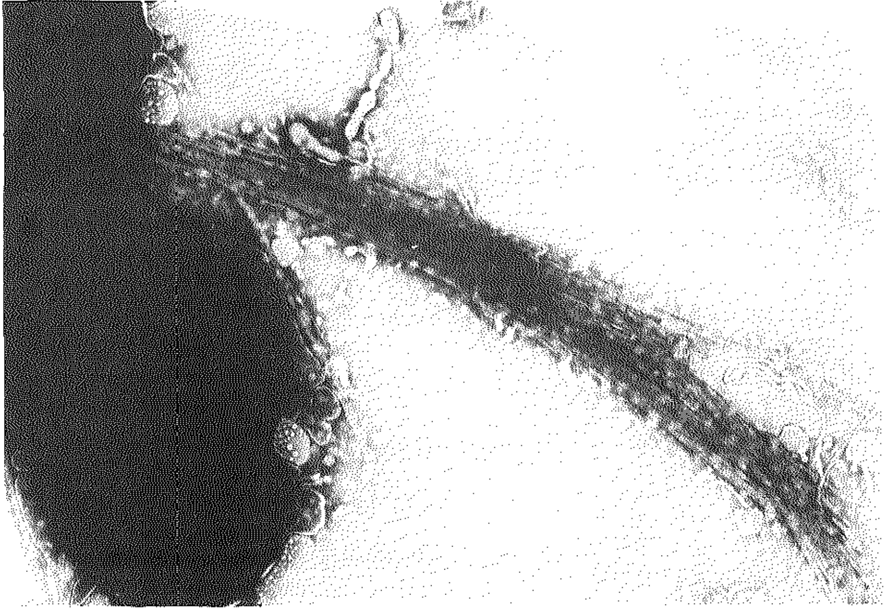
Gonococci were cultured and checked for the correct type as in the above described method. The gonococci from 150 Kellogg plates were harvested in ice-cold (0°C) 0.01 M Tris HCl buffer pH 9.5. The harvest from 75 plates was suspended in 25 ml. The homogenization and the check-up by Gram staining were in accordance with the previous method. The pili were then separated from the bacteria by centrifuging suspensions at 12,000 x g during 10 minutes and at 50,000 x g during 1 hour. After combining the supernatants in one cylinder (about 45 ml), the same volume of 20% saturated $(NH_4)_2SO_4$ (ice-cold, 0°C) was added to precipitate the pili. This mixture had to be gently stirred during about 14 hours (overnight) at 4°C. The next day it was centrifuged at 30,000 x g during 15 minutes. The supernatant was carefully removed by suction. The pilus precipitate was re-suspended in 0.5 - 1 ml 0.01 M Tris HCl buffer pH 7.0 (with 0.01 M NaN_3 added) and stored at 4°C. The quality was checked electron-microscopically, and insufficiently pure pilus preparations were rejected.

Characterization of pilus antigen

Gonococci and pilus preparations were assessed electron-microscopically in a Philips EM300 microscope at the Department of Cell biology of the Erasmus University, with the technical assistance and advice of P. J. Visser. The samples to be tested were negatively stained with 2% sodium phosphotungstate pH 7.6 (photograph 1 and 2) using carbon-impregnated collodium copper grids (mesh 200). All pilus preparations were found to be slightly polluted by vesicles (possibly endotoxin blebs or cell membrane fragments). The protein content of the pilus preparation was determined according to Lowry et al.[3], using bovine serum albumin as standard.

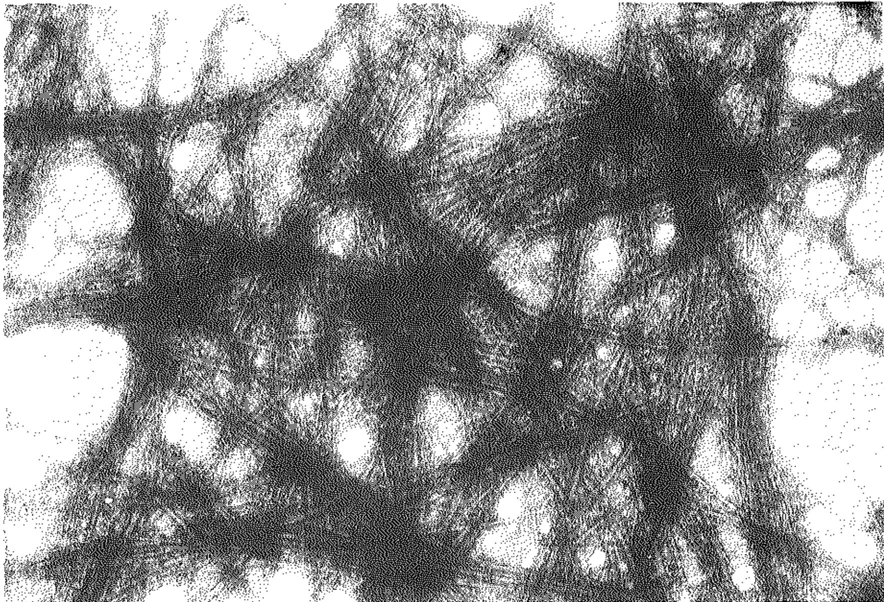
ELISA test principle

The ELISA is a highly sensitive serological system in which an enzyme is linked to antibodies or antigens[4,5]. Such an enzyme/antibody or enzyme/antigen conjugate has both immunological and enzymatic activity. The reaction between antigen and antibody takes place on the wall of tubes and microtitre plates. The most widely used enzymes are peroxidase or alkaline phosphatase. A substrate suitable to the enzyme used is offered, and the ultimate result is a change of colour. The intensity of this change of colour can be determined with



Photograph 1:
Electron micrograph of a highly piliated gonococcus, (colony type 2, strain 1443), negatively stained 23300 x.

Photograph 2:
Electron micrograph of gonococcal pili purified from strain 6650, 29300 x.

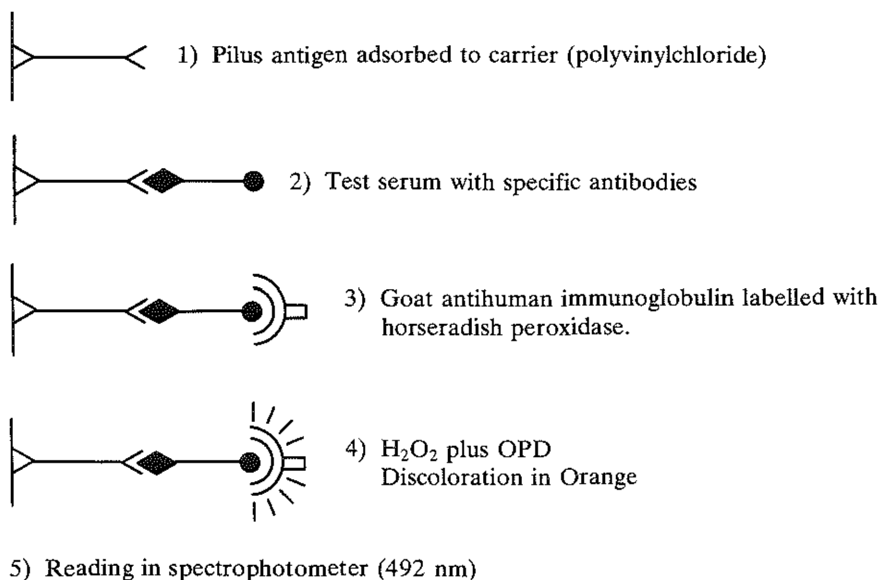


the naked eye or, more exactly and more objectively, by measuring the absorbance in a spectrophotometer[6].

ELISA procedure

The various steps of the procedure used in this study are schematically represented in figure 1. This is known as the indirect ELISA.

Figure 1 *Schematic representation of the ELISA*



Reagents

PBS pH 7.8: 10 tablets Dulbecco A were dissolved in 1 l distilled water and autoclaved at 115°C for 10 minutes. One ampoule Dulbecco B was added to the solution after cooling.

Sodium carbonate buffer: 35 ml 0.1 M $NaHCO_3$, 65 ml 0.1 M Na_2CO_3 and 20 mg NaN_3, adjusted to pH 9.6.

Buffer pH 5.0: equal amounts of 0.2 M Na_2HPO_4 and 0.1 M citric acid, adjusted to pH 5.0.

Substrate solution: 40 mg orthophenylene diamine was dissolved in 100 ml buffer (pH 5.0) and 40 μl 30% H_2O_2 was added to the solution.

Assay procedure

Coating with antigen:

The pilus antigen required, expressed in μg/ml protein[3], was supplemented with sodium carbonate buffer pH 9.6 to a total volume of 2 ml in a small MSE 65 tube. Immediately before use the antigen was sonicated in an ultrasonic disintegrator (8 micron, probe 3,3 mm) during 1 minute at 0°C. After sonication the antigen was further diluted to the concentration required in the test. This pilus antigen concentration was established by chessboard titration and varied from 2 μg to 6 μg protein/ml, dependent on the pilus batch.

The microtitre plates were coated by depositing 50 μl pilus antigen in each cup; incubation was carried out for 4 hours at 37°C in a water bath. All other incubations were carried out under the same conditions. After washing with PBS pH 7.8, 100 μl 1% bovine serum albumin was added and the plates were re-incubated for 30 minutes at 37°C to cover any free reaction sites. After further washing with PBS pH 7.8, the plates were stored at 4°C.

Assay:

Patient sera were diluted 1:100 in PBS pH 7.8, and 50 μl aliquots of the diluted serum were added (in duplicate) to the cups and incubated for 45 minutes at 37°C. The plates were then washed four times with PBS pH 7.8 (plus 0.05% Tween 20), and 50 μl of an optimal dilution of horseradish peroxidase-conjugated antihuman total immunoglobulin (Institut Pasteur) was added. The plates were then re-incubated for 45 minutes at 37°C and washed six times with PBS pH 7.8 (plus 0.05% Tween 20). After addition of 50 μl substrate the plates were incubated for 50 minutes at 37°C. The reaction was stopped by adding 50 μl 2M H_2SO_4. A positive and a negative serum were included on each microtitre plate. A row of antigen controls (coated cups without serum) and a maximum colour control (conjugate plus substrate) were likewise included on each plate.

Absorbance was read at 492 nm using a Multiskan (Titertek) spectrophotometer, with reference to a row of blanks (antigen controls).

Test conditions

The optimal conjugate dilution was established by antigen conjugate titrations in order to minimize non-specific coloration.

The optimal antigen concentration was established exactly by performing the assay with a strongly positive, a weakly positive and a negative serum on cups coated with different antigen concentrations.

The duration of passive adsorption ("coating") of the antigen to the polyvinylchloride microtitre plates (flat bottom PVC, Cooke microtitre system) was varied in time and temperature: 16 hours at 4° and 24, 6, 5, 4, 3 and 2 hours at 37°C. These times were chosen in view of data from the literature[6] and for

practical reasons. Again, the three previously mentioned reference sera were used. The optimal coloration and change of colour took place between 3 and 5 hours at 37°C. The "coating" time was set at 4 hours at 37°C. "After-coating" with 1% bovine serum albumin enhanced the evenness of coloration but to some extent also the background.

The abovementioned antigen conjugate titration was repeated for each series of coated plates. The antigen-coated plates could be stored for 10 days at 4°C after washing them once with PBS pH 7.8; longer storage entailed a gradual loss of quality.

Reliability and precision of the ELISA results

In an effort to establish the reliability and precision of the results in the test system, the following experiments were set up.

1) The variation "within one microtitre plate" was examined by testing all cups of the plate with the same serum.
2) The "day-to-day" variation was examined by testing simultaneously antigen-coated microtitre plates daily with two sera.
3) The variation between different coatings was examined by comparing the results obtained with 20 sera on plates with four different coatings: A, B, C and D.

All these experiments were performed with the ELISA, using gonococcal pili 1443 as antigen. All the results of these experiments were accounted for in the "results".

re 1) *Variation "within one plate"*

In early experiments, a positive serum was tested eight-fold in serial serum dilutions on the horizontal rows of a microtitre plate.

Per vertical row (same serum dilution) the result was assessed qualitatively with the naked eye; four independent investigators assessed all rows as showing no difference.

In subsequent experiments this test was repeated in a serum with a high absorbance result; in view of the serum strength, this was done at serum dilution 1:400. The mean absorbance of eight determinations was 1.16, the standard error being 0.07 and the coefficient of variation being 6%.

It can be concluded that variation "within one plate" was inconsiderable.

re 2) *Day-to-day variation*

Two sera were tested on microtitre plates on four consecutive days, the four microtitre plates having been coated simultaneously with the same antigen concentration. The first serum was pooled from some positive sera (P), and the second from negative and weakly positive sera (D). Absorbance results, standard errors and coefficients of variation of the sera are presented in table 1.

Table 1 *Day-to-day variation in sera P and D*

Pool sera	Serum dilution 1:100			Serum dilution 1:200		
	mA*	SE	CV%	mA	SE	CV%
P	1.76	0.16	9	1.29	0.17	13
D	0.75	0.18	24	0.58	0.11	19

* mean absorbance.

The coefficient of variation in the positive serum was less than 15%, and about twice that of variation "within one plate." In the serum with a low absorbance value the coefficient of variation was higher, the standard error remaining constant.

re 3) *Variation between different coatings*

The influence of different coatings on the absorbance result was measured in 20 sera (dilution 1:100). Of these 20 sera, nine had a mean absorbance of 1.50, seven one of 1.00-1.50 and four one of less than 1.00. Sera with a high absorbance value showed a low coefficient of variation (CV $< 15\%$). Inversely, sera with a low absorbance value showed a high coefficient of variation (CV $\geqslant 30\%$). Thirteen of the sera showed a CV of less than 15%. The standard error was virtually the same in all the sera. The results are presented in table 2.

Table 2 *Influence of coating on the variation of the absorbance results in 20 tested sera (dilution 1:100)*

Coating	CV% (median value)
A	19
B	12
C	8
D	13
A, B, C and D together	13

To summarize: the coefficient of variation in sera with a high absorbance value was less than 15%, while sera with a low absorbance value showed coefficients of variation of 30% or more. A striking feature of this experiment, too, was the constancy of the standard error ($\triangle A = \pm 0.15$). There were virtually no differences in variation between this experiment and the "day-to-day variation" experiment; the influence of different coatings was therefore small.

Conclusion from experiments 1 through 3

These experiments demonstrate that the ELISA as performed in this study had a constant standard error ($\Delta A = \pm 0.15$). This led to a low coefficient of variation at high absorbances, and to a high coefficient of variation at low absorbances.

In view of these results we formulated as requirement for the definitive performance of the ELISA that the absorbance results may show a coefficient of variation of no more than 15%. In actual practice this meant that 5-10% of all assay runs had to be rejected. This requirement was not applied to sera with very low absorbance values (evidently negative).

Choice of the breakpoint between positive and negative in the ELISA

The determination of the breakpoint between positive and negative, and the problems this poses, are discussed in the appendix to chapter 5.

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CHAPTER 5

Enzyme-Linked Immunosorbent Assay (ELISA) Using Gonococcal Pili as Antigen:

The value of testing consecutive serum samples obtained from defined patient groups attending an STD Clinic in Rotterdam.

Arnold P. Oranje*, Annet de Roo**, Ernst Stolz* and Marc F. Michel**
Department of Dermatology and Venereology*,
Clinical Microbiology and Antimicrobial Therapy**, Erasmus University Rotterdam.

Summary

An ELISA system was developed which uses gonococcal pili as antigen. The value of this test was studied by applying it to paired serum samples (the first taken at the first examination, and the second 11-22 days later) from certain patients. The patient groups studied were strictly defined and selected. Particular attention was paid to the contribution of the examination of the second sample to the test sensitivity of the assay. Testing of the second sample did not add to test sensitivity in asymptomatically infected females. For males and females with uncomplicated gonorrhoea, however, the testing of a second paired serum sample led to a marked increase of sensitivity; this was also observed for infected males with 5-30 days of symptoms.

Maximum sensitivity was 53% in the total group of infected females, 61% in the asymptomatic females, 41% in the total male group, and 62% in the males with 5-30 days of symptoms.

When applied to sera from patients with oropharyngeal gonorrhoea the ELISA attained up to 96% sensitivity. This study confirms previous findings that serological tests using gonococcal pili as antigen are unsuitable as a diagnostic tool unless oropharyngeal gonorrhoea or complicated gonococcal infections are suspected.

Introduction

Numerous serological tests for detection of gonorrhoea have been described[1]. The best known and most widely used of these is the GCFT.[2-6] This test, however, has the disadvantage of being neither very sensitive nor very specific.

1. To be published as an abstract in the Proceedings of the 224th Meeting of the Netherlands Society for Dermatology and Venereology, Rotterdam. February 1982.
2. Complete paper accepted for publication in the European J. Sex. Transm. Dis.

Buchanan et al.[7] described a RIA which uses gonococcal pili as antigen; they attained a high level of sensitivity with this test, especially in asymptomatically infected females (89%). Oates et al.[8], Reimann et al.[9], Brinton et al.[10], and Young and Low[11] confirmed the excellent antigenic properties of gonococcal pili in a RIA[8], an IHA[9] and an ELISA[10, 11].

This article presents results obtained with an ELISA system which uses pili from a single strain of *Neisseria gonorrhoeae* as antigen. This system was applied to sera from selected, well-defined patient groups with and without gonorrhoea, attending an STD clinic in Rotterdam. Special attention was paid to the contribution to the sensitivity of the assay made by examination of a second, consecutive serum sample taken 11-22 days after the first examination.

Material and Methods

Patients

Between September 1977 and July 1978, sera were obtained from patients with and without gonorrhoea attending an STD clinic at the University Hospital in Rotterdam. Samples were taken from cervix, urethra, rectum and oropharynx of females and from urethra, oropharynx and rectum (only if indicated) of males. Each specimen was cultured on selective Thayer-Martin medium[12] and on chocolate agar medium without antibiotics. Suspected oxidase-positive colonies were identified as gonococci by Gram stain and sugar fermentation reactions. If cultures of first and a second examination (after 1-2 weeks) were negative, the patient in question was considered not to be suffering from gonorrhoea. A positive Gram stain and negative cultures for gonorrhoea were found in only a few patients, who were excluded from this study. All patients were subjected to routine tests for detection of other STD as described by Stolz[13].

Generally patients were included in the study only if two consecutive serum samples could be obtained at an interval of 11-22 days. They were then assigned to the following groups:

Patients with gonorrhoea

- females with uncomplicated urogenital/rectal gonorrhoea (n=104), subdivided into those with asymptomatic (n=56) and those with symptomatic infections (n=48);
- males with uncomplicated urogenital/rectal gonorrhoea (n=129), subdivided into those with 1-4 days of symptoms (n=79), those with 5-30 days of symptoms (n=34), and those who fell into neither of these two groups (n=16).
- patients with oropharyngeal gonorrhoea (n=24)

Patients without gonorrhoea:

- females attending the clinic for an STD check (n=28);
- female prostitutes (n=21);

– males attending the clinic for an STD check (n=72);
– males with non gonococcal urethritis (n=83).
Control sera were obtained from 119 blood donors (46 females, age range 19-65 years, and 73 males, age range 20-62 years) and 58 children (25 girls and 33 boys aged between 10 and 12 years).

Sera from blood donors were kindly donated by Dr F. Kothe of the Rotterdam Transfusion Service, and sera from children by Prof. Dr H. A. Valkenburg and Dr F. Klein of the Department of Epidemiology, Erasmus University Rotterdam. The children in question were all healthy inhabitants of Zoetermeer (a suburb of The Hague).

Buffers and growth media

GC medium: Difco medium, 36 g/l, supplemented with 2% Isovitalex.
Liquid medium: 4.5 g proteose peptone, 0.3 g K_2HPO_4, 1.5 g NaCl, 30 ml glycerol, and 300 ml distilled water. (For further details see chapter 4 page 14.)

Gonococcal strain

A stable highly piliated strain, 1443, isolated in Rotterdam in 1976, was used for pilus production.

Preparation of gonococcal pilus antigen

Type 2 gonococci of strain 1443 were cultured on solid growth media. The inoculated plates were incubated at 37°C for 18 to 20 hours in moist air containing 5% CO_2 (in a CO_2 incubator type F 303, Assab, Sweden). The pili were purified as described by Robertson et al.[14] (with some minor modifications). For details see chapter 4, page 11-12.

Characterization of pilus antigen (chapter 4, page 12)[15].

ELISA procedure

Coating and assay: see chapter 4, page 14-15.

Reading: All sera were tested only at dilution 1:100. Absorbance was read at 492 nm using a Multiskan (Titertek) spectrophotometer, with reference to the row of blanks. Readings were classified as follows: $A \geqslant 0.90$ "positive", absorbance between 0.80 and 0.90 were classified as "doubtful", and those between 0.90 and 1.00 as "weak-positive". All sera from the children tested, had absorbances below 0.90.

The coefficient of variation within the test (plate to plate) and from test to test (day to day) was less than 15%; only for undoubtedly negative sera was it higher.

The first and second serum samples were tested as a pair; all sera including the controls, were tested in duplicate.

Statistical analysis

Fisher's test[16] was used to compare the percentages of positive results in two different patient groups; the two-sided tail probability was computed in the way proposed by Cox and Hinkley[17].

McNemar's test[16] was used to compare the percentages of positive results in two consecutive serum samples from the same patients.

Results

Patients with uncomplicated gonorrhoea

Table 1 lists the percentages of ELISA-positive sera in the various groups of patients with gonorrhoea. In the group of females with uncomplicated gonorrhoea (n=104), 45% of the first serum samples and 53% of those taken 11-22 days later were positive, the difference being nearly significant. When these 104 females were subdivided into those with asymptomatic infection (n=56), and those with symptomatic infection (n=48), the sensitivity of the ELISA was – as expected – higher in the former group (D_1= 57%, D_2= 61%) than in the latter (D_1= 31%, D_2= 44%). The difference between the two D_1 values is significant.

For females with asymptomatic infection the sensitivity was not significantly higher in the second serum sample than in the first one. In those with symptomatic infection there was a difference in sensitivity, but not a significant one. Cumulative sensitivity was 58% in the total group of females with uncomplicated gonorrhoea, the corresponding figures in the subgroup with asymptomatic and that with symptomatic infection being 66% and 48% respectively.

For males with uncomplicated gonorrhoea (n=129), 32% of the first serum samples and 41% of those taken 11-22 days later were positive. The males with uncomplicated urogenital/rectal gonorrhoea were subdivided into a group with 1-4 days of symptoms (n=79), one with 5-30 days of symptoms (n=34), and a small residual group with more than 30 days of symptoms or with no established record of symptoms (n=16). The sensitivity of the ELISA was higher in the group with 5-30 days of symptoms (D_1= 44%, D_2= 62%) than in that with 1-4 days of symptoms (D_1= 25%, D_2= 34%). The difference between the two D_1 values is nearly significant and that between the two D_2 values is significant. In the total group of males with uncomplicated gonorrhoea and in those with 5-30 days of symptoms the sensitivities on D_2 were significantly higher than those on D_1. Cumulative sensitivity was 47% for males with uncomplicated gonorrhoea, the corresponding figures in the subgroup with 1-4 days of symptoms and in that with 5-30 days of symptoms being 42% and 62% respectively.

Oropharyngeal gonorrhoea

Paired serum samples were obtained from 24 patients in this group. The ELISA results in this group are presented in table 2: 21 of the 24 sera were

Table 1 *Sensitivity of ELISA, using gonococcal pili 1443 as antigen, in different patient groups on date of first examination (D_1) 11-22 days after first examination (D_2) and on D_1 and/or D_2 (cumulative)*

Patient groups	Sensitivity %		
	D_1	D_2	Cumulative
1. FEMALES with current GC (n=104)	45	53	58
a. asymptomatic uncomplicated urogenital/rectal GC (n=56)	57	61	66
b. symptomatic uncomplicated urogenital/rectal GC (n=48)	31	44	48
2. MALES with current GC (n=129)	32	41	47
a. with uncomplicated urogenital/rectal GC with complaints for 1-4 days (n=79)	25	34	42
b. with uncomplicated urogenital/rectal GC with complaints for 5-30 days (n=34)	44	62	62
c. others (n=16)	37	31	37

Significance of differences in sensitivity on D_1 and D_2 between the different groups, assessed by Mc Nemar's test

Patient groups*	Comparison of D_2 with D_1
Group 1	$0.05 < p < 0.1$
Group 2	$p < 0.05$
Group 2_b	$p < 0.05$

Significance of sensitivity differences observed between the various groups, as assessed by Fisher's test

Groups compared	D_1	D_2
1_a and 1_b*	$p = 0.01$	ns
2_a and 2_b	$p = 0.07$	$p = 0.007$

* only relevant groups are mentioned.

positive on D_1, and 23 of the 24 sera were positive on D_2 (6-22 days after D_1). Test sensitivity was therefore 87% on D_1, 96% on D_2 and cumulative 100%.

Table 2 *Sensitivity of ELISA using gonococcal pili 1443 as antigen in patients with oropharyngeal gonorrhoea, on date of first examination (D_1), 6-22 days after first examination (D_2) and on D_1 and/or D_2 (cumulative)*

Patient groups	Sensitivity %		
	D_1	D_2	Cumulative
Total group	87 (21)*	96 (23)*	100 (24)
– females (n=15)	87 (13)	93 (14)	100 (15)
– males (n=9)	89 (8)	100 (9)	100 (9)

* Absolute numbers are given in brackets.

Patient groups without gonorrhoea

Table 3 shows that test specificity in patients without gonorrhoea varied widely from group to group.

The highest specificity was found in males who attended the clinic for an STD check (D_1= 87%, D_2= 90%), and it was nearly as high in the males with NGU (D_1= 84%, D_2= 83%).

Specificity in females visiting the clinic for an STD check was somewhat lower (D_1= 75%, D_2= 82%) and prostitutes attending the clinic showed the lowest specificity (D_1= 52%, D_2= 38%).

Table 3 *Specificity of ELISA, using gonococcal pili 1443 as antigen, in different patient groups on date of first examination (D_1), 11-22 days after first examination (D_2) and on D_1 and/or D_2 (cumulative)*

Patient groups	Specificity %		
	D_1	D_2	Cumulative
1. FEMALES without current GC (n=49)			
a. requesting an STD check (n=28)	75	82	64
b. prostitutes (n=21)	52	38	29
2. MALES without current GC (n=155)			
a. requesting an STD check (n=72)	87	90	85
b. with NGU (n=83)	84	83	82

Group 1_a scored significantly higher than group 1_b on D_2 ($p = 0.003$ Fisher's test); the cumulative specificity difference between these groups is significant too ($p = 0.02$ Fisher's test).

Cumulative specificity was appreciably lower in the groups with cultures negative for gonorrhoea: 64% in females and 85% in males visiting the clinic for an STD check. The prostitutes showed a particularly low cumulative specificity: 29%. Cumulative specificity in males with NGU pratically equalled that in males requesting an STD check: 82% versus 85%.

Other groups

As already mentioned, sera from 119 blood donors and from 58 children aged 10-12 years were included in this study. No positive results were obtained in either of these groups. Two doubtful results were read in the blood donor groups, and four in the children. Of the two sera from children with meningococcal sepsis, one was strongly positive (A=1.50), while the other was negative (A=0.34).

Discussion

The ELISA originally developed by Engvall and Perlman[18] and Van Weemen and Schuurs[19] has proved to be of value in various serological studies. Not only does this system permit measurement of very low antibody levels but it also has the advantage of lending itself to a high degree of mechanization, thus avoiding human errors and possible faulty readings[20].

Our version of the ELISA, using gonococcal pili as antigen, gave the best results in infected females (particularly in those with asymptomatic infections), in patients with oropharyngeal gonorrhoea and in patients with complicated gonorrhoeal infections (see chapter 6).

The observed sensitivities and specificities would seem to warrant the conclusion that the ELISA in its present form is not suitable as an aid in the diagnosis of gonorrhoea in a patient population of an STD clinic. The short incubation time of gonorrhoea is probably the principal cause of the poor sensitivity of the assay. Serological gonorrhoea tests such as the ELISA are generally suitable for case detection in patients suffering from non-genital localized STD.

It is important that a serological test is both sensitive and specific. For the purpose of case detection and screening, moreover, it is important (for practical and economic reasons) that only one blood sample need be examined. With the ELISA we used, we found that in the second serum sample tested the sensitivity was higher in certain groups of patients, e.g. females with symptomatic gonorrhoea, and all male patients examined. In the latter total group the increase in sensitivity was significant. However, most males suffer from symptomatic gonorrhoea of short duration, which can be diagnosed quite well on the basis of the clinical signs and hardly requires the use of a serological test.

The results obtained in asymptomatically infected females clearly show that the use of two serum samples does not ensure a significantly higher score than the use of one sample in tracing unnoticed gonorrhoea of long duration.

The diagnosis of complicated gonococcal infections and oropharyngeal

gonorrhoea is a potential area of application for this ELISA. In oropharyngeal gonorrhoea we achieved a very high sensitivity.

Taking the results on D_1 and on D_2 cumulatively, we find that sensitivity increases in all groups, whereas specificity decreases (as it can be expected to do).

We think that, in the ELISA performed by us goncoccal pili may cross-react with antibodies evoked by other species of *Neisseria*. Four of the 58 sera from children showed absorbances which we classified as "doubtful". Moreover, the ELISA gave a strong positive result with one of the two sera from children with meningococcal sepsis. Buchanan et al.[7] likewise found increased levels of antibodies against gonococcal pili in three adult patients with meningococcal infections. Such cross-reactions with meningococcal antibodies are a well-known phenomenon in serological tests for gonococcal antibodies[10, 21, 22].

In the ELISA we use antihuman total immunoglobulin-labelled horseradish peroxidase as conjugate. The choice of immunoglobulin class-specific conjugates might make it possible to distinguish between recent and previous gonorrhoea, but unfortunately we were unable to measure any antibody response of significance with antihuman IgA and IgM conjugates. Only a few of the sera of 79 males with recent uncomplicated gonorrhoea showed an IgM response (unpublished data). The immune response to gonococcal pili in human beings is dominated by IgG antibodies, as previously also found by Reimann et al.[9]

It might be possible to enhance the sensitivity of the ELISA by adding one or more pilus antigens to the present pilus preparation.

Given a positive ELISA result, a bacteriological culture should always be made before treatment is instituted. It is also important to question the patient about a possible history of gonorrhoea, for a positive result could also be due to previous gonorrhoea (whether treated or not).

The ELISA is a suitable tool for the diagnosis of oropharyngeal gonorrhoea and complicated gonococcal infections. In the lastmentioned group it is not always possible to obtain a positive gonococcal culture and in such cases it would be justifiable to give treatment solely on the basis of a positive serological test.

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Appendix

There are two methods of interpreting the result of a serological assay. The first calls for determination of a breakpoint for the distinction between a positive and a negative result. The breakpoint is related on rather arbitrary grounds to the study of a reasonable number of control sera. In the ELISA system the breakpoint can be expressed in several ways, e.g. as a titre of a conventional series of serum dilutions, as the ratio between a positive and a negative result, and by using a breakpoint in the reading of the colour intensity at a particular serum dilution[1].

The second method interprets the test on the basis of disease prevalences expected a priori or a posteriori in certain patient populations[2].

This study has opted for a system in which the absorbance values of sera are determined at one fixed dilution of 1:100. Sensitivity and specificity data related to a fixed breakpoint, however, do not give sufficient insight into the effectiveness of a test in certain patient populations.

A serological test can be used for screening or case-detecting purposes; the purpose for which a test is used determines in part how many false positive and/or false negative results will be accepted, i.e. where the breakpoint between positive and negative is set. The purpose of this discussion is to gain more insight into the effectiveness of an ELISA with gonococcal pili 1443 as antigen as screening or case-detecting test for gonorrhoea. To achieve this, the results obtained in patient populations with and without uncomplicated gonorrhoea were first interpreted in relation to three absorbance values: $A \geqslant 0.80$, $A \geqslant 0.90$ and $A \geqslant 1.00$. Next, the possible test results in three imaginary population groups or patient populations with gonorrhoea prevalences of 0.1%, 3% and 30% are discussed. Sensitivity, specificity and predictive values of positive and negative test results are terms used in the interpretation of the data.

Patients with uncomplicated gonorrhoea

Table 1 lists the percentage of positive sera obtained from a number of males and females with uncomplicated gonorrhoea when different breakpoints are used in the interpretation of the ELISA. As expected, sensitivity increased when the breakpoint was lowered. The highest sensitivity was attained in females with uncomplicated gonorrhoea: 56% at $A \geqslant 0.80$.

Patients without gonorrhoea

Table 2 indicates the specificity of the test in a number of males and females without gonorrhoea who attended an STD clinic for a check-up. The specificity logically decreased when the breakpoint was lowered. The specificity was lowest in the group of females (n=28): 57% at $A \geqslant 0.80$. At that level it was still high in the group of males (n=72): 83%.

Table 1 *The influence of different breakpoints on the sensitivity of the ELISA using gonococcal pili 1443 as antigen in patients with uncomplicated gonococcal infections attending an STD clinic*

Patient groups	Sensitivity %		
	A ⩾ 0.80	A ⩾ 0.90	A ⩾ 1.00
1. FEMALES with current GC (n = 104)	56	45	41
2. MALES with current GC (n = 129)	39	32	22

Results relate to sera obtained on the day of the first examination.

Table 2 *The influence of different breakpoints on the specificity of the ELISA using gonococcal pili 1443 as antigen in patients without current gonococcal infections attending an STD clinic, and in two control groups*

Patient groups	Specificity %		
	A ⩾ 0.80	A ⩾ 0.90	A ⩾ 1.00
1. FEMALES without current GC, with request for STD check-up (n = 28)	57	75	89
2. MALES without current GC, with request for STD check-up (n = 72)	83	87	94
3. BLOOD DONORS (n = 119)	98	100	100
4. CHILDREN aged 10-12 years (n = 58)	93	100	100

Results relate to sera obtained on the day of the first examination.

Blood donors and children

Table 2 also lists the results obtained in 119 blood donors and 58 children aged 10-12 years. At breakpoint A ⩾ 0.80, the specificity was 98% in the blood donors and 93% in the children. At the other breakpoints the specificity attained a value of 100%.

Predictive value of the ELISA in different population groups and patient populations

Table 3 lists the predictive values of a positive and a negative test result. As an example, the calculations on a female population with a prevalence of 0.1% at breakpoint A ⩾ 0.80 will be discussed in detail.

A population with a gonorrhoea prevalence of 0.1% (e.g. the entire Neth-

Table 3 *Predictive values of a positive and a negative test result at different gonorrhoea prevalences*

Prevalence	Sex	Breakpoint					
		A ⩾ 0.80		A ⩾ 0.90		A ⩾ 1.00	
		PV(+)	PV(−)	PV(+)	PV(−)	PV(+)	PV(−)
0.1%	F*	0.03	1.00	1.00	1.00	1.00	1.00
	M	0.02	1.00	1.00	1.00	1.00	1.00
3%	F	0.04	0.98	0.05	0.98	0.10	0.98
	M	0.07	0.98	0.07	0.98	0.10	0.97
30%	F	0.36	0.75	0.44	0.76	0.61	0.78
	M	0.50	0.76	0.51	0.75	0.61	0.74

* F = female, M = male.

erlands population, or females who undergo an abortion) is a low-risk group[3]. The blood donors can best serve as a measure of the specificity in this population. At A ⩾ 0.80, sensitivity is 56% and specificity is 98% (table 1 and 2). Let the number of females examined be 100.000, then 100 of them are suffering from gonorrhoea. Of these 100 females with gonorrhoea, 56 have a positive and 44 have a negative test. The 99.900 females without gonorrhoea include 97.902 with a negative and 1998 with a positive test. Of the 56 + 1998 = 2054 with a positive test, 56 have gonorrhoea, i.e. PV(+) = 56/2054 = 0.03. Of the 44 + 97.902 = 97, 946 females with a negative test, 97,902 are free from gonorrhoea, i.e. PV(−) = 97.902/97.946 = 1.00. More or less the same figures apply to the males. Both in the males and in the females, the predictive values at A ⩾ 0.90 and A ⩾ 1.00 are virtually the same: 1.00 for a positive and 1.00 for a negative result.

The predictive values for populations with gonorrhoea prevalences of 3% and 30% are calculated in exactly the same way and are likewise listed in table 3. In populations with a prevalence of 30% (as can be expected at an STD clinic) the test is unsatisfactory at A ⩾ 0.80 and A ⩾ 0.90. Only at A ⩾ 1.00 do the predictive values begin to assume an acceptable level: at this absorbance value the predictive value of a positive result is 0.61, while that of a negative result is 0.78 in females and 0.74 in males. This implies that a positive test result in females is correct in 61% of the cases, but incorrect (false positive) in 39%. A negative test result is correct in 78% of the cases, but incorrect (false negative) in 22%.

If at an STD clinic 200 female patients are examined with the aid of the ELISA (A ⩾ 1.00), then 60 females (30%) can be expected to suffer from

gonorrhoea and 140 can be expected to be free from it. The ELISA result will therefore be positive in [0.41 (sensitivity) x 60 = 25] + [0.11 (1-specificity) x 140 = 15] = 40 cases, and negative in 160. Of these results, 15 are false positive and 35 are false negative. This means that 50 (25%) of the 200 serum samples examined have given rise to an erroneous diagnosis or postulate.

Galen and Gambino[4] formulated the following criteria for serological tests used in screening for gonorrhoea: sensitivity should be 80% or higher, specificity should be 99% or higher, and no positive results should be found in patients with a history of gonococcal infection.

The results clearly show that the ELISA using gonococcal pili 1443 as antigen is not suitable as a screening test in groups with a low gonorrhoea prevalence of 0.1% or 3%. In a population with a high gonorrhoea prevalence of 30% (as can be expected in patients attending an STD clinic) the test is not satisfactory with $A \geqslant 0.80$ or $A \geqslant 0.90$ as breakpoint absorbance value. At a breakpoint of $A \geqslant 1.00$ the predictive values of the assay begin to attain acceptable levels in such a population, albeit that the assay result is still erroneous – i.e. false negative or false positive – in 25% of the cases. It should be borne in mind, moreover, that the sensitivity of the assay diminishes markedly at absorbance value $A \geqslant 1.00$. In order to ensure nevertheless a reasonable balance between sensitivity and specificity, the following compromise was accepted. The breakpoint absorbance value was set at $A \geqslant 0.90$. Results with absorbance values $0.90 \leqslant A < 1.00$ were called weakly positive. At results with absorbance values $0.80 \leqslant A < 1.00$, the assay should be repeated in a fresh serum sample from the patient in question.

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CHAPTER 6

ELISA and IHA using two different gonococcal pili as antigens and GCFT using whole gonococci as antigen:

Comparison of results obtaind by testing sera from patients attending an STD clinic in Rotterdam and from control groups.

Arnold P. Oranje*, Karin Reimann**, Ron V. W. van Eijk***, Hubert J. A. Schouten+, Annet de Roo**, Gerdy J. Tideman***, Ernst Stolz* and Marc F Michel**.

* Department of Dermatology and Venereology, Erasmus University Rotterdam.
** Department of Clinical Microbiology and Antimicrobial Therapy, Erasmus University Rotterdam.
*** Laboratory of Bacteriology, National Institute of Public Health, Bilthoven.
\+ Institute of Biostatistics, Erasmus University Rotterdam.

Summary

Three serological tests for detection of gonococcal antibodies were compared: an ELISA, an IHA and a GCFT. The ELISA was performed with gonococcal pili of a Rotterdam strain (1443), the IHA with pili of an American strain (2686, Buchanan), and the GCFT with whole gonococci of a single strain (46695, Oliver). The tests were applied to sera from several groups of Dutch patients. Serum samples were taken at the first examination (D_1) and generally 11-22 days later (D_2).

The ELISA and the IHA were far more sensitive than the GCFT. The specificities of the tests were virtually equal in low-risk groups, but in high-risk groups the GCFT seemed slightly more specific. The ELISA and the IHA virtually did not differ in sensitivity and specificity. Maximal sensitivity for females with uncomplicated gonorrhoea was 53% for the ELISA, 58% for the IHA and 22% for the GCFT. The corresponding sensitivities in asymptomatically infected females were 61%, 63% and 21%, respectively. In the infected

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2. Presented at the meeting of the Netherlands Society for Microbiology Maastricht, May 1982.
3. To be published as an excerpt in Antonie van Leeuwenhoek.

males with 5-30 days of symptoms the sensitivities were practically as high as those in the asymptomatically infected females. Maximal sensitivity for males with uncomplicated gonorrhoea was 41% for the ELISA, 50% for the IHA and 14% for the GCFT.

The agreement between ELISA and IHA for patients with uncomplicated gonorrhoea was surprisingly not very high (kappa = 0.44), but as could be expected the agreement between GCFT and the two pilus assays was decidedly less (kappa = 0.26 and 0.20). The highest sensitivities were obtained with all three tests when they were applied to sera from patients with oropharyngeal gonorrhoea and complicated gonococcal infections. Again the ELISA and the IHA were more sensitive than the GCFT.

In their present form, serological tests using gonococcal pili as antigen are of value as a diagnostic tool only in cases of suspected gonococcal complications. The value of the GCFT, described here, is very limited.

Introduction

The most interesting gonococcal antigens for use in serological tests and application as vaccine are at this time pili and outer membrane protein complexes[1-5]. Buchanan et al. (1973)[2] were the first to publish promising results obtained with a RIA using gonococcal pili of strain 2686 (USA) as antigen. In sera from males and females with uncomplicated gonorrhoea, the sensitivity was 50% and 86%, respectively.

Reimann et al.[6] obtained almost equally good results with an IHA using a mixture of gonococcal pili of two strains.

We developed an ELISA in which gonococcal pili of a Rotterdam strain (1443) are used as antigen[7].

This paper compares the results of the ELISA with those of the IHA using gonococcal pili of Buchanan's strain 2686, and with the GCFT routinely used in The Netherlands, in which whole gonococci of a single strain (Oliver) are used as antigen.

Material and Methods

Patients

For details see chapter 5, page 20-21 [8,9].

Generally patients were included in the study only if two consecutive serum samples could be obtained at an interval of 11-22 days. They were then assigned to the following groups:

Patients with gonorrhoea

- females with uncomplicated urogenital/rectal gonorrhoea (n=104).
- males with uncomplicated urogenital/rectal gonorrhoea (n=129).
- patients with oropharyngeal gonorrhoea (n=33)
- patients with complicated gonococcal infections (n=11).

Six of the patients were suffering from a disseminated gonococcal infection,

one from bartholinitis, three from epidydimitis and one from a rectal abcess.

Patients without gonorrhoea:

- females attending the clinic for an STD check (n=28);
- female prostitutes (n=21);
- males attending the clinic for an STD check (n=72);
- males with NGU (n=83).

All patients were also asked whether they had previously contracted gonorrhoea; the various groups defined above could thus be further subdivided into those with a positive and those with a negative history of gonorrhoea.

Control sera were obtained from 119 blood donors and 58 children aged between 10 and 12 years.

ELISA

The preparation of the gonococcal pilus antigen of strain 1443 (Rotterdam) and the procedure of the ELISA have been previously described[7]. Pilus antigen was purified according to the method of Robertson et al.[10]. Serum samples which showed an absorbance $\geqslant 0.90$ were regarded as positive. (See chapter 4 and 5 for more details.)

IHA

The IHA was carried out as previously described by Reimann and Lind[11], using purified gonococcal pili of Buchanan's strain 2686 (USA) as antigen. Pilus antigen was purified according to the method of Hermodson et al[12]. The titre of a given serum specimen was recorded as the reciprocal value of the lowest serum concentration giving a positive reaction. Titres $\geqslant 40$ were considered to be positive.

GCFT

The GCFT was performed as a mechanized microtechnique using Ames Autotiter instrument. Briefly, 0.05 ml inactivated serum was automatically added to the U-shaped cups of clear Conphar trays and diluted in two-fold dilutions from undiluted to 1 : 512 in 0.85% saline. Antigen (0.025 ml) and complement (0.05 ml) were then added to each cup and the reagents were incubated overnight at 4°C. The next morning, 0.025 ml each of amboceptor and 2% v/v sheep red blood cells suspended in saline were automatically dispersed into each cup. The trays were shaken and incubated at 37°C for 1 hour. The titre was read as the highest dilution giving 100% inhibition of lysis, if the control cup showed 100% haemolysis. Serum showing less than 100% haemolysis in the control cup was regarded as anticomplementary. About 5% of the sera in this patient material were anticomplementary and thus could not be read.

The antigen used in the GCFT test was prepared from *Neisseria gonorrhoeae* strain 46695 (Oliver) by ultrasonic treatment of whole organisms suspended in saline[13].

Statistical analysis

Fisher's test[14] was used to compare the percentages of positive results in two different patient groups; the two-sided tail probability was computed in the way proposed by Cox and Hinkley[15]. McNemar's test[14] was used to compare the percentages of positive results in two consecutive serum samples from the same patients. The coefficient kappa[16, 17] was used to measure the agreement between two serological tests. Kappa equals zero under statistical independence and kappa equals one when there is perfect agreement.

Results

Patients with uncomplicated gonorrhoea

Table 1 lists the percentages of positive sera in the ELISA, IHA and GCFT in patients with uncomplicated gonorrhoea.

Of the sera obtained for females with uncomplicated gonorrhoea (n=104). 45% were positive in the ELISA, 58% in the IHA and 21% in the GCFT on the day of the first examination (D_1). The corresponding sensitivities of the sera obtained 11-22 days later (D_2) were 53%, 54% and 22%, respectively. When D_2 is compared with D_1, a nearly significant increase in percentage of positive sera is observed only in the ELISA. No significant differences in sensitivity are found between ELISA and IHA. The difference of both tests from the GCFT, however, is significant both on D_1 and on D_2.

For females with asymptomatic infections (n=56), the sensitivities of ELISA, IHA and GCFT on D_1 were 57%, 63% and 21%, respectively, versus 61%, 55% and 18%, respectively, on D_2. Comparison of sensitivities on D_2 with those on D_1 reveals no significant differences for any of the three tests. In fact the IHA and GCFT had lower sensitivities on D_2. ELISA and IHA show no significant difference in this group. The difference between these two tests and the GCFT, however, is again significant both on D_1 and on D_2.

For females with symptomatic infections the respective sensitivities of ELISA, IHA and GCFT were 31%, 52% and 21% on D_1 and 44%, 52% and 27% on D_2. ELISA and IHA sensitivities differed significantly on D_1 but not on D_2. ELISA and GCFT differed significantly on D_2 but not on D_1. IHA and GCFT differed significantly both on D_1 and on D_2.

ELISA and IHA sensitivities were both higher in the group of asymptomatically infected than in that of symptomatically infected females. This does not apply to the GCFT. In fact, on D_2 the sensitivity in the asymptomatic was lower than that in the symptomatic group (18% versus 27%). Only in the ELISA on D_1 did the sensitivity of these two groups differ significantly.

Of the sera obtained for males with uncomplicated gonorrhoea (n=129), 32% were positive in the ELISA, 36% in the IHA and 10% in the GCFT. The corresponding sensitivities on D_2 were 41%, 50% and 14%, respectively. When D_2 is compared with D_1, both the ELISA and the IHA show a significant increase in percentage of positive results. The difference in GCFT sensitivities

Table 1 *Sensitivities of three serological tests in patients with uncomplicated gonococcal infections attending an STD Clinic*

Patient groups	Sensitivity %		
	ELISA	IHA	GCFT

Table 2 *Specificities of three serological tests in patients without current gonococcal infections attending an STD clinic*

Patient groups	Specificity %					
	ELISA		IHA		GCFT	
	D1	D2	D1	D2	D1	D2
1. FEMALES without current GC						
a. Reporting for STD check-up (n=28)	75	82*	71	71	96	96
b. prostitutes (n=21)	52	38*	52	48	86	91
2. MALES without current GC						
a. Reporting for STD check-up (n=72)	87	90	82	82	93	96
b. With NGU (n=83)	84	83	75	76	90	94

Table 2 *(continued)*
P values when the ELISA, IHA and GCFT results are compared using McNemar's test

Comparison		P values in group			
		1a	1b	2a	2b
ELISA versus IHA	D1	ns	ns	ns	$0.05<p<0.10$
	D2	ns	ns	ns	ns
GCFT versus ELISA	D1	$0.05<p<010$	$p<0.05$	ns	ns
	D2	ns	$p<0.01$	ns	$p<0.05$
GCFT versus IHA	D1	$p<0.05$	$p<0.05$	$p<0.05$	$p<0.01$
	D2	$p<0.05$	$p<0.01$	$p<0.01$	$p<0.01$

* On D2 for ELISA results: group 1a versus group 1b $p=0.003$ (Fisher's test).

is not significant. ELISA and IHA did not differ significantly in this total group of males. Both were significantly more sensitive than the GCFT.

For males with 1-4 days of symptoms (n=79) the ELISA, IHA and GCFT sensitivities were 25%, 27% and 4%, respectively, on D_1, versus 34%, 39% and 10%, respectively, on D_2. Comparison of the sensitivity on D_2 with that on D_1 reveals a significant increase in the percentage of positive sera only in the IHA. Comparison of the three tests gives the same result as that in the total group of males.

For males with 5-30 days of symptoms (n=34) the ELISA, IHA and GCFT sensitivities were 44%, 53% and 23%, respectively, on D_1 versus 62%, 77% and 23%, respectively, on D_2. Comparison of the sensitivity on D_2 with that on D_1 reveals a significant increase in percentage of positive sera in both the ELISA and the IHA. Only in this subgroup of males did ELISA and IHA sensitivities differ significantly on D_2. The difference in sensitivity between ELISA and GCFT was significant only on D_2. IHA and GCFT differed significantly both on D_1 and on D_2, as they did in the total group of males.

Comparison of sera from males with 1-4 days with those from males with 5-30 days of symptoms reveals higher sensitivities of all tests in the latter subgroup, the difference nearly always being significant.

Patients without gonorrhoea

Table 2 shows the specificities of the three tests for patients without gonorrhoea.

In females reporting for an STD check-up (n=28) the ELISA, IHA and GCFT specificities were 75%, 71% and 96%, respectively, on D_1, versus 82%, 71% and 96%, respectively, on D_2. ELISA and IHA specificities did not differ significantly. ELISA and GCFT showed a near-significant difference in specificity only on D_1 (75% versus 96%). IHA and GCFT differed significantly both on D_1 and on D_2 (71% versus 96%).

The specificities of all tests were lowest for the prostitutes (n=21). The specificities of ELISA, IHA and GCFT were 52%, 52% and 86%, respectively, on D_1, versus 38%, 48% and 91%, respectively, on D_2. Again, ELISA and IHA did not differ significantly; both tests, however, were significantly less specific than the GCFT.

In males reporting for an STD check-up (n=72) the specificities of the ELISA, IHA and GCFT were 87%, 82% and 93%, respectively, on D_1, the corresponding specificities on D_2 being 90%, 82% and 96%, respectively. Again, ELISA and IHA specificities did not differ significantly. The same applies to ELISA and GCFT. Comparison between GCFT and IHA, however, reveals a higher specificity of GCFT. Test specificity for males with NGU was slightly lower than that in the preceding group. ELISA, IHA and GCFT scored 84%, 75% and 90%, respectively, on D_1, versus 83%, 76% and 94%, respectively, on D_2. In this group the difference between ELISA and IHA on D_1 just escapes significance (84% versus 75%). Only on D_2 did the GCFT show a

Tabel 4 *(continued)*
P values when test results (sensitivity) in groups with and without previous GC are compared using Fisher's test

Comparison		ELISA	IHA	GCFT
group 1_a versus 1_b	D1	ns	p=0.02	ns
	D2	ns	ns	ns
group 2_a versus 2_b	D1	ns	p=0.006	p=0.07*
	D2	ns	p=0.05	ns

* in reversed direction: group 2_b versus 2_a.

is recorded, only on D_2 for females the increase is not significant. For gonorrhoea patients with a record of previous gonorrhoea it is remarkable that the percentage of positive sera in the GCFT is even smaller than that in the groups without previous gonorrhoea.

Comparing the different groups of patients without gonorrhoea (table 5), we

Table 5 *The effect of previous gonococcal infection(s) on the specificities of three serological tests for detection of gonococcal antibodies*

Patient groups	Specificity %					
	ELISA		IHA		GCFT	
	D1	D2	D1	D2	D1	D2
1. FEMALES without current GC (n=49)						
a. with previous GC (n=28)	61	46	46	43	89	93
b. without previous GC (n=21)	71	86	86	86	95	95
2. MALES without current GC (n=155)						
a. with previous GC (n=43)	77	74	70	72	86	88
b. without previous GC (n=112)	89	91	81	81	96	98

Table 5 *(continued)*
P values when test results (specificity) in groups with and without previous GC are compared using Fisher's test

Comparison		ELISA	IHA	GCFT
group 1_b versus 1_a	D1	ns	p=0.007	ns
	D2	p=0.007	p=0.003	ns
group 2_b versus 2_a	D1	p=0.07	ns	p=0.07
	D2	p=0.02	ns	p=0.02

find a higher specificity of all tests in the groups without a record of previous gonorrhoea. The three tests all show a (partly significant) difference in this respect.

Other groups (table 6)

The specificity of all three tests is high in the groups of blood donors (n=119) and children (n=58).

Table 6 *Specificities of three serological tests for detection of gonococcal antibodies in blood donors and children*

Groups	Specificity %		
	ELISA	IHA	GCFT
Blood donors (n=119)	100	94	99
Children aged 10-12 years (n=58)	100	100	100

Supplemental information obtained by combining the tests

Tables 7 and 8 list the results of the tests, separately and in combination.

Table 7 shows the sensitivity in females with uncomplicated gonorrhoea (n=104). The highest sensitivity is of course attained when the results of all three tests are combined: 68% positive sera on D_1 and 70% on D_2. The percentage of positive sera with the combined ELISA and/or IHA results virtually equals that with the three tests combined: 67% on D_1 and 69% on D_2.

Table 7 *Results obtained by using one or more of three serological tests for detection of gonococcal antibodies in 104 females with uncomplicated urogenital/rectal gonorrhoea*

Test or combination of tests	Sensitivity %	
	D1	D2
ELISA	45	53
IHA	58	54
GCFT	21	22
ELISA and/or GCFT	52	56
IHA and/or GCFT	61	59
ELISA and/or IHA	67	69
ELISA and/or IHA and/or GCFT	68	70

Table 8 shows the sensitivity in males with uncomplicated gonorrhoea (n=129). The highest sensitivity is of course again attained when the results of the three tests are combined: 46% positive sera on D_1 and 60% on D_2. As in the females, the percentage of positive sera with the combined ELISA and/or IHA results virtually equals the sensitivity attained with the combination of the three tests: 44% on D_1 and 59% on D_2.

Specificity decreases accordingly when the test results are combined.

Table 8 *Results obtained by using one or more of three serological tests for detection of gonococcal antibodies in 129 males with uncomplicated urogenital/rectal gonorrhoea*

Test or combination of tests	Sensitivity %	
	D1	D2
ELISA	32	41
IHA	36	50
GCFT	10	14
ELISA and/or GCFT	33	45
IHA and/or GCFT	38	52
ELISA and/or IHA	44	59
ELISA and/or IHA and/or GCFT	46	60

Degree of agreement between the tests

The agreement between the tests is measured with the aid of the coefficient kappa. Table 9 presents the agreement between the results of the ELISA, IHA and GCFT; 466 examinations were performed in 233 patients with uncomplicated gonorrhoea (two samples from each patient).

By way of example we present a comparison between ELISA and IHA in detail. Both testst were performed N=466 times. The number of agreeing results observed was: $n_o = 146 + 191 = 337$. The number of agreeing results expected in the case of statistical independence is: $n_c = 95 + 140 = 235$. The expected number is calculated exactly as in a chi-square test. When the two tests agree only by chance, the expected and the observed numbers are about equal. In the case under consideration the observed number of agreeing results exceeds the expected number: $n_o - n_c = 337 - 235 = 102$. The maximum possible difference is: $N - n_c = 466 - 235 = 231$ (when all results of both tests are the same). The ratio $(n_o - n_c)/(N - n_c)$ is known as the coefficient of agreement kappa. In this case kappa = 102/231 = 0.44.

For the agreement between ELISA and GCFT kappa = 0.26. Nearly the same result is obtained when IHA and GCFT are compared, namely kappa = 0.20.

Table 9 *Agreement between three serological tests for detection of gonococcal antibodies in 233 patients with uncomplicated gonorrhoea on the date of first examination (D1) and 11-22 days later (D2)*

		IHA +	IHA −	
ELISA	+	146 (95)	50	kappa=0.44
	−	79	191 (140)	SE=0.04

		GCFT +	GCFT −	
ELISA	+	59 (32)	137	kappa=0.26
	−	17	253 (226)	SE=0.04

		GCFT +	GCFT −	
IHA	+	60 (37)	165	kappa=0.20
	−	16	225 (202)	SE=0.03

\+ = positive, − = negative.
The number which can be expected under statistical independence is given in brackets.

Discussion

Many serological tests for detection of antibodies to gonorrhoea have been developed. However, it is difficult to compare the various results because the tests were performed under different conditions. The culture technique, the sites from which the cultures were taken and the selection of patient populations differ widely in the different studies. Few studies have compared two or more serological tests in the same patient population[6, 18-20].

Danielsson et al.[20] compared a GCFT and an immuno-electrophoresis test (IE) in the same, well-defined patient groups. The GCFT scored 20-25% in males and 30-40% in females with uncomplicated gonorrhoea. The sensitivity of the IE was 24% in patients (male and female) with uncomplicated gonorrhoea.

Reimann et al.[6] compared an IHA test using a mixture of pili from two Danish gonococcal strains as antigen with the GCFT (routine test in Denmark until 1979). The sensitivity of the IHA test was 70% for females and 59% for males. The GCFT sensitivity was 9% in both groups.

We performed a comparative study of three tests: an ELISA and IHA, using

two different gonococcal pili as antigen, and a GCFT using whole gonococcal cells as antigen.

We found the highest ELISA and IHA sensitivities in patients with complicated gonococcal infections and oropharyngeal gonorrhoea, in asymptomatically infected females, and in males with 5-30 days of symptoms of uncomplicated gonorrhoea. The sensitivity of the GCFT was very low in the uncomplicated infections. In uncomplicated gonorrhoea the highest sensitivity (27%) was attained on D_2 (11-22 days after the first examination) for females with symptomatic infections. The sensitivities were much higher in complicated and oropharyngeal gonorrhoea, although they were still lower than those of ELISA and IHA. In oropharyngeal gonorrhoea the sensitivity of the ELISA was significantly higher than that of the GCFT, and near-significantly higher than that of the IHA. Only for symptomatically infected females and males with 5-30 days of symptoms of uncomplicated gonorrhoea did ELISA and IHA differ significantly in sensitivity (IHA being superior). The ELISA would generally seem to be slightly more specific than the IHA, but its sensitivity seemed to be slightly lower except in oropharyngeal gonorrhoea, where it was superior to IHA.

Using the coefficient of agreement kappa, we found a higher degree of agreement between ELISA and IHA (kappa = 0.44) than between each of these tests and the GCFT (kappa = 0.26 and 0.20). The ELISA and IHA systems have virtually the same sensitivity, but the coefficient of agreement in our study is nevertheless lower than might be expected. The unexpectedly low agreement may be due to different antigenic determinants on the two pilus antigens. But this can only partly explain the relatively low agreement. Another but improbable possibility is that different components of the pili are available as antigen in each system.

Reimann et al. (1982)[21] recently reported that antigenic heterogeneity of gonococcal pili exists if human sera are used in the test system (chapter 7). It turned out that pili of strain 6650 (Rotterdam) had a broader antigenicity in the IHA than the pili of Buchanan's strain 2686. In females with uncomplicated gonorrhoea (same sera as in this study) Reimann et al.[21] attained sensitivities of 66% and 71% on D_1 and D_2, respectively. Oranje et al. (chapter 8), using an ELISA performed with pili of strain 6650 in the same patient population, attained virtually the same results: 69% on D_1 and 67% on D_2.

The two pilus tests are far more sensitive than the GCFT, but the specificity of the latter is higher in high-risk groups. The low specificity of the pilus tests in these groups is partly due to the large proportion of patients with a history of previous gonococcal infections. Holmes et al.[22], Reimann et al.[11] and the present authors found that antibodies to gonococcal pili are mostly of the IgG class and can therefore persist very long (sometimes for years). Not infrequently, some patients attending STD clinics may, without being aware of it, cure their gonococcal infection with antibiotics they already have in stock. Culture will then be negative but the serological test result may be positive.

The persistence of antibodies to gonococcal pili and (partly unsupervised) use of antibiotics also explain the low specificity of the tests in prostitutes. Moreover, the sensitivity of the gonococcal cultures is not 100% either. For example, the ELISA as applied in the prostitutes revealed three patients from whom no gonococci could be cultured but who showed a marked increase in absorbance within two weeks – indicating a current or a very recent infection. For proper evaluation of a serological test for detection of gonorrhoea, therefore, insight into the incidence of false negative gonococcal culture results is of great importance.

It is of little use to combine one of the two pilus tests with the GCFT. When the ELISA and IHA results are combined, a sensitivity is attained which nearly equals the combined sensitivity of the three tests. The value of the GCFT as performed in this study, is exceedingly limited.

The findings warrant the conclusion that gonococcal pili constitute an excellent antigen, which may be improved by including antigenically different pilus antigens in the antigen preparation used, or by replacing the antigen with a more broad-spectred pilus antigen.

A positive serological reaction should always be confirmed with positive cultures or positive clinical symptoms (complicated gonorrhoea) before the diagnosis gonorrhoea is put forward and treatment started. The pilus tests with their relatively high sensitivities may be suitable for case-detecting and screening.

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CHAPTER 7

Demonstration of Antigenic Heterogeneity of *Neisseria Gonorrhoeae* Pilus antigens using human sera in the test system

Karin Reimann*, Arnold P. Oranje** and Marc F. Michel*

Department of Clinical Microbiology and Antimicrobial Therapy* and
Department of Dermatology and Venereology**, Erasmus University Rotterdam.

Summary

Antigenic heterogeneity of gonococcal pili using human sera in the test system was demonstrated by means of indirect haemagglutination inhibition. The reactivities of sera from 104 females with current gonococcal infection were compared by the IHA test using different pilus preparations as antigens.

Pili were purified from 22 strains infecting patients who met the following three requirements: 1) no history of previous gonococcal infection(s), 2) an infection that has lasted a week or more, 3) a seronegative result when tested by the IHA test using pili from Buchanan's strain 2686 as antigen. Each patient's serum was tested against pili produced by the gonococcal strain infecting this particular patient. A seropositive reaction obtained by using pilus antigen from the strain infecting the seronegative (strain 2686) patient indicates an antigenically heterogeneous pilus preparation. Two such strains, antigenically different from one another and from strain 2686, were found among 22 pilus preparations studied.

Introduction

Several serological tests using gonococcal pili as antigen have been described[1-4]. The results obtained for human sera have been uniform and independent both of the test system and of the strain(s) used for pilus preparation[5]. This suggests that man recognizes very homologous determinants on gonococcal pili as opposed to rabbits[6]. However, none of the serological tests described has a sensitivity higher than 89% (asymptomatically infected females)[1]. The

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2. Present address of first author: Karin Reimann, Neisseria Department, Statens seruminstitut, 80 Amager Boulevard, DK-2300 Copenhagen S. Denmark.
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missing percentages could be due to: 1) an infection too brief to stimulate increased antibody levels to pili, 2) a lack of ability in the patient to develop pilus antibodies or 3) a lack of antigenic determinants in the pilus antigen used to recognize the pilus antibodies generated by the infecting gonococcal strain. This last possibility was investigated by testing individual human sera by means of the IHA test using pilus antigen prepared from the gonococcal strain causing the infection.

Our hypothesis was that once antigenic heterogeneity of gonococcal pili was proven in human sera, the sensitivity of tests to detect antibody to pili in patients with gonorrhoea may be increased by including more than one antigenic type of pili in the antigen preparations.

Materials and Methods

IHA test using gonococcal pili as antigen

The test was performed as described earlier[7] with a few important and timesaving modifications: a) prior to use the pilus antigen was sonicated for one minute at 0°C (8 micron using an MSE ultrasonic disintegrator, probe 3.3 mm), b) none of the sera was inactivated, nor was any absorbed with formalin-treated sheep red blood cells (SRBC), c) the agglutination patterns were read after one hour. The titre of a given specimen was read as the reciprocal value of the highest serum dilution giving a + reaction. Titres ≥ 40 were considered positive but only if the reaction in the first well (serum dilution 1/20) was read as ++ or more.

Haemagglutination inhibition experiments

These studies were performed as described previously[7].

Patients

Sera and correspondent gonococcal strains were collected from 170 female and 195 male patients with current gonococcal infection admitted to the STD clinic at Dijkzigt Hospital, Rotterdam, in the period September 1977-July 1978. All sera were studied by the IHA test for gonococcal pilus antibodies, using pili from Buchanan's strain 2686 as antigen. We selected sera and gonococcal strains from these patients who met the following three requirements: 1) no history of past gonococcal infection(s), 2) an infection that had lasted a week or more before sampling, 3) a seronegative result when tested by the IHA using pili 2686 as antigen. A total of 28 patients fulfilled these three conditions. Pilus antigen was purified from 22 of the strains causing gonococcal infection according to the method of Hermodson et al.[8]. No pili could be prepared from the remaining six strains for various reasons.

Results

Selection of Study Group

Table 1 summarizes the eleven female and the eleven male patients fulfilling the criteria given in Materials and Methods. Each patient's serum was tested against the specific antigenic type of pili produced by the gonococcal strain infecting this particular patient. Our hypothesis was that a seropositive reaction obtained with a patient's own pili in an otherwise seronegative patient would indicate an immunological disparity between the two pilus antigens. By only including patients without a previous gonococcal infection we could be

Table 1 *IHA test performed on sera from 22 patients with current gonococcal infection without previous GC using pili from the infecting strains as antigens*

Patient sera			*IHA titre obtained by using pilus antigen:*		
Sex	*No.*	*No. of days with infection*	*from infecting strain*	*6650**	*30605***
M[Δ]	1	7 - 30	***	.⁺	.
M	2	7 - 30	–	.	.
M	3	7 - 30	–	.	.
M	4	unknown	–	.	.
M	5	7 - 30	80	–	80
M	6	⩾30	–	.	.
M	7	⩾30	–	.	.
M	8	7 - 30	–	.	.
M	9	unknown	–	.	.
M	10	7 - 30	–	.	.
M	11	⩾30	–	.	.
F[Δ]	12	7 - 30	–	.	.
F	13	unknown	–	.	.
F	14	7 - 30	–	.	.
F	15	unknown	–	.	.
F	16	7 - 30	–	.	.
F	17	⩾30	80	80	160
F	18	⩾30	160	80	160
F	19	unknown	–	.	.
F	20	⩾30	–	.	.
F	21	7 - 30	80	80	80
F	22	7 - 30	320	320	640

* 6650 is the strain causing gonococcal infection in patient F22.
** 30605 is the strain causing gonococcal infection in patient M5.
*** – = IHA titre is negative (⩽20).
⁺ . = not done.
Δ M = male, F = female.

certain that the pilus antibodies found were really induced by the strain causing the present infection. Table 1 shows five patients – M5, F17, F18, F21 and F22 – who demonstrated a positive antibody response when pili made from the infecting strain were used as antigen. Strain 6650, cultured from the rectum of patient F22, was by far the best pilus antigen producer and was thus selected for further investigations. Sera from patients M5, F17, F18 and F21 were re-tested, this time using pili 6650 as antigen; all sera except that from patient M5 were positive. This suggested that pili from strain 30605 infecting patient M5 possessed yet a third set of antigenic determinants. Again the sera from the interesting patients – F17, F18, F21 and F22 – were tried by the IHA test using pili produced by strain 30605 as antigen. All sera gave a positive antibody response, which meant that no more antigenically different pili could be found in this material. Pili from strain 30605 were not investigated further in this study; the pilus production of this strain was only half the production of strain 6650. It should be noticed that the heterologous titres and the homologous titres are of the same magnitude when the same human serum was being tested, thus indicating that in case of common antigenic determinants the shared cross-reactivity was 100%.

Haemagglutination Inhibition Studies

Tables 2 and 3 show two haemagglutination inhibition assays reaffirming the antigenic difference between pili from strain 2686 and strain 6650 when using human sera in the test system. Table 2 summarizes an experiment where SRBC (sheep red blood cells), sensitized with pili 2686, were used as antigen; the inhibitors were pili 2686 and pili 6650. The sera studied were "pool serum" (a pool of eight positive sera) giving a positive reaction when tested against both pili 2686 and pili 6650, and "serum A" giving a positive reaction only when tested against pili 2686. The reaction in the "pool serum" was inhibited both by addition of pili 2686 and pili 6650 one hour prior to addition of the sensitized blood. The reaction in "serum A" was inhibited only by addition of pili 2686; no inhibition at all could be detected when pili 6650 up to a concentration of 0.4 μg/ml were added.

Table 3 shows a similar experiment. The antigen in this assay was SRBC sensitized with pili 6650, the inhibitors were the same as in the former experiment and the sera were "pool serum" and "serum B", the latter being positive only when pili 6650 were used as antigen. The reactions in the "pool serum" were inhibited by both pilus preparations, but the reaction in "serum B" was inhibited only when pili 6650 were added and not by addition of pili 2686.

Comparison of results obtained by using the two heterogeneous pilus antigens in the IHA test

In order to establish whether the sensitivity of the IHA test could be improved, we tested a group of females with current uncomplicated urogenital/rectal gonorrhoea (56 with asymptomatic and 48 with symptomatic infec-

Table 2 *IHA test using pili 2686 (0.5 μg/ml) as antigen and using both pili 2686 and pili 6650 as inhibitors*

	IHA titre obtained by testing			IHA titre obtained by testing	
inhibitor pili 2686 μg/ml	pool serum*	serum A**	inhibitor pili 6650 μg/ml	pool serum*	serum A**
0.0	160	160	0.0	160	160
0.025	160	160	0.025	160	160
0.05	160	160	0.05	160	160
0.1	80	80	0.1	80	160
0.2	40	40	0.2	40	160
0.4	–***	–	0.4	–	160

* pool serum: giving a positive antibody response when tested by IHA using both pili 2686 and pili 6650 as antigens.

** serum A: giving a positive antibody response when tested by IHA using pili 2686 as antigen but a negative result if pili 6650 were used as antigen.

*** IHA titre is negative ($\leqslant$20).

Table 3 *IHA test using pili 6650 (0.5 μg/ml) as antigen and using both pili 2686 and pili 6650 as inhibitors*

	IHA titre obtained by testing			IHA titre obtained by testing	
inhibitor pili 2686 μg/ml	pool serum*	serum B**	inhibitor pili 6650 μ g/ml	pool serum*	serum B**
0.0	320	80	0.0	320	80
0.025	160	80	0.025	160	80
0.05	80	80	0.05	80	40
0.1	40	80	0.1	40	–
0.2	–***	80	0.2	–	–
0.4	–	80	0.4	–	–

* pool serum: giving a positive antibody response when tested by IHA using both pili 2686 and pili 6650 as antigens.

** serum B: giving a positive antibody response when tested by IHA using pili 6650 as antigen but a negative result if pili 2686 were used as antigen.

*** IHA titre is negative ($\leqslant$20).

Table 4 *Sera from 104 females with current gonococcal infection tested by the IHA test using pili 2686, pili 6650 and pilus pool (2686 + 6650) as antigens*

Pilus antigen	Sensitivity % when testing patient group					
	56 females with asymptomatic GC		48 females with symptomatic GC		Total	
	D_1	D_2	D_1	D_2	D_1	D_2
2686*	63	55	52	52	58	54
6650*	73	75	58	67	66	71
pool (2686 + 6650)**	79	77	65	71	72	74

* concentration: 0.5 μg/ml.
** concentration: 1.0 μg/ml.

Table 5 *P-values (McNemar's test) for differences in sensitivity using pili 2686, pili 6650 and pilus pool (2686 + 6650) as antigens (based on the results shown in table 4)*

Comparison	56 females with asymptomatic GC		48 females with symptomatic GC		Total	
	D_1	D_2	D_1	D_2	D_1	D_2
6650 versus 2686	ns	$p<0.01$	ns	$0.05<p<0.10$	$0.05<p<0.10$	$p<0.01$
pool (2686+6650) versus 2686	ns	$p<0.01$	$0.05<p<0.10$	$p<0.01$	$p<0.01$	$p<0.01$
pool (2686+6650) versus 6650	ns	ns	ns	ns	ns	ns

tion) using pili 2686, pili 6650 and a pool of pili (2686 + 6650) as antigen (Tables 4 and 5). Blood samples were drawn from all patients on D1: date of first examination and on D2: 11-22 days after D1. The results obtained by using pili 2686 as antigen were compared with the results found by using pili 6650 as antigen (McNemar's test[9]). Likewise, we compared the results by using pili 6650 as antigen with the results obtained when a pool of the two pilus preparations 2686 + 6650 was used as antigen. No significant differences were found in this last comparison, but when the sensitivities found with pilus antigen 2686 were compared with those attained with pilus antigen 6650 and pilus pool (2686 + 6650), a significant improvement was measured using the latter two pilus antigens.

In order to evaluate a possible difference in specificity of the IHA test using the different pilus preparations as antigens, we tested sera from 119 blood donors as well as from 58 healthy children aged 10-12 years. The results shown in Table 6 indicate no significant decrease in specificity when using pili 6650 or pilus pool (2686 + 6650) as antigen instead of pili 2686.

Table 6 *Sera from 119 blood donors and 58 children aged 10-12 years tested by the IHA test using pili 2686, pili 6650 and pilus pool (2686 + 6650) as antigens*

Pilus antigen	Specificity % when testing	
	119 blood donors	58 children aged 10-12 years
2686*	94	100
6650*	92	100
pool (2686 + 6650)**	92	100

* concentration: 0.5 μg/ml.
** concentration: 1.0 μg/ml.

Discussion

The properties of gonococcal pili and their antigenic relationship have been reviewed by Buchanan[10]. A high degree of heterogeneity has been reported when guinea-pig serum or rabbit serum was used in the test systems[11-15]. Novotny and Cownley[15] found no general low-level serological cross-reactivity between pili from 50 gonococcal strains if sera from rabbits or guinea-pigs immunized with whole cells were used in their test system, immune electron microscopy. Buchanan[10] reported that shared antigens accounted for 2.5% or less when the antigenic structure of pili from four different gonococcal strains was investigated using rabbit antibodies to purified pili in the RIA system. Brinton et al.[11] also used rabbit antisera to gonococcal pili. Depending upon the test system used, they found a shared cross-reactivity of 5-15% between 46

gonococcal pilus serotypes, i.e. rabbit antiserum heterologous titres were approximately 5-15% of the homologous titres.

On the other hand serological tests using gonococcal pili as antigen[1-4] have, when performed on sera from patients, exhibited sensitivities far above what could be expected, had human sera recognized the same antigenic determinants as rabbit sera. This apparent difference in cross-reactivity prompted the study by Lind and Reimann[6] in which rabbit antisera to pili and sera from seropositive patients were used in IHA inhibition experiments. Pili from four strains were tested; only two strains possessed common antigens detectable by means of rabbit antisera (weak cross-reactivity), but all four cross-reacted 100% when human sera were used in the system. Brinton et al.[11] have reported nearly 100% cross-reactivity among 50 pilus preparations from arbitrarily chosen strains when sera from naturally diseased humans were used in the test system.

In order to establish whether antigenic heterogeneity existed or not, we could use only sera and strains from patients without a history of previous gonorrhoea, i.e. we only wanted to demonstrate antibodies stimulated by pili from the currently infecting strain. Sera and strains from 28 patients were therefore selected according to the criteria described in Materials and Methods, and pilus antigen was purified from 22 of these strains. If a patient was seronegative also when tested against pilus antigen from the infecting strain, we concluded that the infection time before sampling was too short for formation of a detectable level of antibodies or that the patient lacked the ability to generate pilus antibodies. Five of the 22 patient sera gave a positive antibody response when tested against pili produced by the infecting strain. Two of these strains possessed pili whose antigenic determinants differed from one another and from those of pili 2686.

Strain 6650 (being by far the best pilus antigen producer) was used in a study where we compared the sensitivities obtained by using pili 2686 alone, pili 6650 alone or a pool of pili (2686 + 6650) as antigens in the IHA test. We found a significant increase in sensitivity of the IHA test if pili 6650 alone or a pool of the two antigenically different pilus preparations (2686 + 6650) were used as antigens instead of using pili 2686 only. No significant difference was found when we compared the results obtained with the pool antigen with those found with pili 6650 alone.

One explanation of the increased sensitivity could be that pili of strain 6650 had a broader spectrum of antigenic determinants than pili of strain 2686. There is also a possibility that antigenic types of pili produced by gonococci differ in different geographic areas, and this may explain why the IHA test using pili from strain 6650 (isolated from a patient from Rotterdam) as antigen, resulted in a significantly higher sensitivity for patients visiting an STD clinic in Rotterdam than when pili of strain 2686 (isolated years ago in the USA) were used.

The heterogeneity of gonococcal pili when human sera were used in the test

system should be borne in mind when selecting suitable antigens for serological tests or for future vaccines.

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CHAPTER 8

Enzyme-linked immunosorbent assay (ELISA) for detection of gonococcal antibodies using two antigenically different gonococcal pili as antigen.

Arnold P. Oranje*, Karin Reimann**, Annet de Roo**, Hubert J. A. Schouten***, Ernst Stolz* and Marc F. Michel**.

* Department of Dermatology and Venereology, Erasmus University, Rotterdam.
** Department of Clinical Microbiology and Antimicrobial Therapy, Erasmus University, Rotterdam.
*** Institute of Biostatistics, Erasmus University, Rotterdam.

Summary

Antibodies to pilus antigens of two gonococcal strains (6650 and 1443) isolated in Rotterdam, were detected in paired sera from females with and without gonorrhoea attending an STD clinic.

The sensitivity of the ELISA using gonococcal pili 6650 as antigen (ELISA 6650) was significantly higher than that attained using gonococcal pili 1443 as antigen (ELISA 1443). The specificity of the two tests hardly differed. On D1 the sensitivity in females with uncomplicated gonorrhoea was 69% in the ELISA 6650 and 45% in the ELISA 1443; the corresponding values in asymptomatically infected females were 75% and 57%, respectively.

The agreement (both in positive and in negative results) between the two tests was less than might be expected (kappa=0.41).

Introduction

Antigenic heterogeneity of gonococcal pili to gonococcal antibodies in human sera was first demonstrated by Reimann et al. (chapter 7)[1] using an IHA. Three antigenically different pilus preparations were demonstrated.

The sensitivity of the IHA using one of these pilus preparations as antigen (6650) was significantly higher than that attained by using the antigenically different pili 2686 as antigen. The sensitivity of the enzyme-linked immunosorbent assay (ELISA) using gonococcal pili 1443 as antigen virtually equalled that of the IHA using gonococcal pili 2686 as antigen (chapter 6)[2]

The aim of this investigation was to establish whether the use of gonococcal pili 6650 as antigen in the ELISA would also increase the sensitivity as compared with that attained with pili 1443 as antigen.

Complete paper accepted for publication in the Brit. J. Vener. Dis.

Material and Methods

We studied sera from 104 females with uncomplicated gonorrhoea and 28 females who requested a check-up for STD. Sera from blood donors (n=119) and from healthy children aged 10-12 (n=58) were used as control sera. All sera used in this study belong to the serum material previously studied in detail. (chapter 5, page 20-21)

Gonococcal strains and preparations of pilus antigen

The gonococcal strains used were 1443 and 6650, both isolated in Rotterdam. Pili 1443 were purified as described by Robertson et al.[3] Pili 6650 were purified according to Hermodson et al.[4] In the former case the gonococci were homogenized and, after differential centrifugation, purified over a caesium chloride gradient. In the latter case gonococci were pre-processed in the same way but purified by precipitation with ammonium sulphate. On the basis of electron-microscopic examinations the pilus preparations looked equally pure.

ELISA

The ELISA procedure was carried out as previously described (chapter 4). In the same way as in the ELISA using gonococcal pili 1443 as antigen (ELISA 1443), the breakpoint in the ELISA with pili 6650 as antigen (ELISA 6650) was determined as $A \geqslant 0.85$. The breakpoint was chosen in such a way that all sera from 58 healthy children aged 10-12 years were completely negative. Results with absorbance values between 0.75 and 0.85 were regarded as dubious, and those with absorbance values between 0.85 and 0.95 as weakly positive in the ELISA 6650. In both assays the coefficient of variation within the test (plate-to-plate) and between tests (day-to-day) was less than 15%; only for unmistakably negative sera was this coefficient higher.

Statistical analysis (see chapter 6, page 36)[5, 6, 7, 8]

Results

Females with uncomplicated gonorrhoea

Table 1 shows the percentage of positive sera in both ELISA tests for females with uncomplicated gonorrhoea (n=104). At the date of the first examination the ELISA 6650 was positive in 69% of the sera, and 11-22 days later this assay was positive in 67% of the sera.

The 104 patients were divided into females with asymptomatic infections (n=56) and females with symptomatic infections (n=48). The sensitivity of the ELISA 6650 was higher in the former (D1: 75%, D2: 70%) than in the latter group (D1: 63%, D2: 65%). This difference is not significant. In neither group did the testing of a second serum sample give any advantage in terms of

Table 1 *Comparison of the sensitivities attained by using pili 6650 and pili 1443 as antigen in the ELISA. Sera from 104 females with uncomplicated gonorrhoea were tested*

	Sensitivity of the ELISA %					
	56 females with asymptomatic GC		48 females with symptomatic GC		total group of 104 females with uncomplicated GC	
	D1	D2	D1	D2	D1	D2
Pili 6650	75	70	63	65	69	67
Pili 1443	57	61	31	44	45	53
McNemar's test	$p < 0.05$	ns	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$

Table 2 *Comparison of the specificities attained by using pili 6650 and pili 1443 as antigen in the ELISA. Sera from females without gonorrhoea, blood donors and children were tested*

	Specificity of the ELISA %			
	28 females without gonorrhoea who requested an STD check-up		119 blood donors	58 children aged 10-12
	D1	D2		
Pili 6650	71	73*	92	100
Pili 1443	75	82	100	100
McNemar's test	ns	ns	$p < 0.01$	ns

* only 26 serum samples tested.

increased sensitivity. Generally, the sensitivity of the ELISA 6650 exceeded that of ELISA 1443. This difference in sensitivity was significant except on D2 in the group of females with asymptomatic infections.

Specificity

Table 2 shows the specificity of ELISA 6650 and ELISA 1443 for females without gonorrhoea who requested a check-up for STD (n=28), for blood donors (n=119) and for healthy children aged 10-12 (n=58.) There was hardly any difference in specificity when using the two antigens. For the blood donors the ELISA 1443 was positive in none of the sera and dubious in 2%, the corresponding figures for the ELISA 6650 being 8% and 5%, respectively.

For the children the ELISA 1443 was dubious in 7% of the sera, while the ELISA 6650 was dubious in none.

Degree of agreement between the tests

The agreement between the tests was measured using the coefficient kappa. Table 3 shows the agreement or dis-agreement between the tests in 208 determinations (N) made in 104 female patients with uncomplicated gonorrhoea. Agreement was observed in 147 tests (n_o). The number of agreements expected under statistical independence is 105 (n_c). The ratio $(n_o-n_c)/(N-n_c)$ is known as the coefficient of agreement kappa. For agreement between the two tests, kappa =(147-105)/(208-105) = 42/103 =0.41.

Table 3 *Agreement between assays using different gonococcal pili (6650 or 1443) as antigen in testing sera from 104 females with uncomplicated gonorrhoea at date of first examination (D1) and 11-22 days later (D2)*

		Pilus 6650 assay +	*Pilus 6650 assay* −	
Pilus 1443 assay	+	93 (72)	12	kappa = 0.41
	−	49	54 (33)	SE = 0.06

\+ = positive.
− = negative.

Number to be expected under statistical independence is given in brackets.

Discussion

Serological tests using gonococcal pili as antigen for detection of gonococcal antibodies have been described in several publications [9-13]. The high sensitivity

attained by Buchanan et al.[10] using a RIA, 86% in females with uncomplicated gonorrhoea, was never attained in any of the other studies[9, 11, 12], except that of Oates et al.[13] for asymptomatically infected females.

Differences in sensitivity attained in the various studies could be due to differences in patient material, to the test system used, but also to the different gonococcal pili used as antigen. Reimann et al. (chapter 7)[1] attained significantly different sensitivities when using pili from strains 6650 and 2686 as antigens in the IHA test performed on the same sera as were used in this study.

This study was undertaken to establish whether the use of pili 6650 as antigen would increase the sensitivity of the ELISA as compared with that attained using pili 1443 as antigen. This was indeed the case (69% with pili 6650 and 45% with pili 1443 on D_1). The specificities hardly differed.

Using the coefficient of agreement kappa, a lower degree of agreement was found between the two versions (kappa = 0.41) than might be expected. In fact this agreement was even less than the kappa (0.44) between the ELISA 1443 and the IHA using gonococcal pili 2686 as antigen (chapter 6)[2]. This is probably entirely due to the heterogeneity of the pilus preparations.

It would be interesting to use a pool of these two or more pilus preparations as antigen in the ELISA. Combination of pili 6650 with pili 2686 in the IHA did not lead to the desired improvement (chapter 7)[1]. The heterogeneity of gonococcal pili when using human sera in the test system is emphasized once more and should be borne in mind when choosing antigens for serological tests or for vaccination.

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CHAPTER 9

Antibodies to gonococcal pili in asymptomatic females

The diagnostic value of testing serum samples by ELISA in females without complaints attending an STD clinic in Rotterdam

Arnold P. Oranje*, Carmen O.A.-Iserief*, Annet de Roo**, Ernst Stolz* and Marc F. Michel**

* Department of Dermatology and Venereology, Erasmus University, Rotterdam.

** Department of Clinical Microbiology and Antimicrobial Therapy, Erasmus University, Rotterdam.

Summary

The diagnostic efficacy of the ELISA using gonococcal pili 6650 as antigen was studied in females without complaints attending an STD clinic for examination and, if necessary, treatment. The total number of females studied was 182, and gonorrhoea was diagnosed in 52 (29%).

The value of the ELISA was calculated on the basis of four arbitrary breakpoints in the test. The best predictive value for a positive result (PV(+)) was obtained at breakpoint $A \geq 1.15$; the best predictive value for a negative result was obtained at breakpoint $A \geq 0.85$.

It is proposed that the ELISA be used as follows. The result is positive at $A \geq 1.15$, negative at $A < 0.85$, the PV(+) then being 0.52 and the PV(−) being 0.85. Whenever sera give a result between $A = 0.85$ and $A = 1.15$, the test should be repeated.

In this version, the ELISA is of some assistance in the diagnosis of gonorrhoea in this group of patients.

Introduction

Females with asymptomatic gonorrhoea constitute a source from which fresh infections can readily arise. Serological tests to detect gonorrhoea which use gonococcal pili as antigen, prove to score high in females with asymptomatic gonococcal infections.[1-5]

Our previous studies showed that the ELISA using gonococcal pili of strain 6650 as antigen attains a sensitivity of 75% in females with asymptomatic urogenital/rectal gonorrhoea (chapter 8).[4]

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This paper discusses the diagnostic efficacy of the ELISA using gonococcal pili 6650 as antigen if several absorbance breakpoints in the test are considered. Sera from females without complaints who attended the STD clinic of the Rotterdam University Hospital Dijkzigt for examination and, if necessary, treatment were tested with the ELISA so interpreted.

Material and Methods

Patients

We collected sera from 182 females without complaints who attended the STD clinic of the Rotterdam University Hospital Dijkzigt between 1st January and 15th December 1981. Professional female prostitutes were excluded. At each visit material was obtained from the cervix, urethra, rectum and oropharynx and tested for gonococci by means of a culture. The specimens were cultured on selective Thayer-Martin[6] medium, and suspect oxidase-positive colonies were identified as gonococci by Gram staining and sugar fermentation tests. When all cultures were negative, the examination was repeated after one week if possible. When all cultures were again negative, the females were considered not to suffer from gonorrhoea. There were no patients with a positive Gram stain and negative cultures. In addition, all patients were routinely tested for the presence of other STD (Stolz)[7].

Gonococcal strain and preparation of pilus antigen

Pili of strain 6650 (Rotterdam) were used to prepare the antigen. They were purified according to Hermodson et al.[8]

ELISA

The ELISA procedure was carried out as already described (chapters 4 and 8).[3,4]

Results

Prevalence of gonorrhoea

Of the 182 females, 52 were suffering from asymptomatic urogenital/rectal gonorrhoea (table 1); one had oropharyngeal gonorrhoea as well. In addition, 26 females reporting as gonorrhoea contacts had gonorrhoea-negative cultures. These patients were as a rule treated on the basis of the history. The remaining 104 patients neither suffered from gonorrhoea nor reported as gonorrhoea contact. The prevalence of gonorrhoea was therefore 52/182=29%.

The patients were divided in addition into those with and those without a history of gonorrhoea (table 1). A history of gonorrhoea existed in 36 of the 182 patients, i.e. 20%. The previous gonococcal infection(s) dated back from a

Table 1 *Classification by diagnosis of 182 asymptomatic females attending an STD clinic*

Patient groups	Number of patients		
	No previous GC	Previous GC	Total
Asymptomatic GC	42	10	52
GC-contact; negative GC cultures	18	8	26
No GC (contact); negative GC cultures	86	18	104
Total	146	36	182

few months to years. The prevalence of gonorrhoea in the group without a history of gonorrhoea was the same as that in the entire group (42/146=29%).

ELISA absorbance values

Figure 1 shows the ELISA absorbance values in the various groups of patients. Accepting the absorbance value A⩾0.85 (initially established as breakpoint in this ELISA)[4] as the boundary between positive and negative, sera from females with asymptomatic gonorrhoea were positive in 38/52=73%; the corresponding figures for gonorrhoea contacts and for the remainder were 13/26=50% and 36/104=35%, respectively. At the various other absorbance breakpoints the corresponding sero-positive ELISA results were calculated (table 2). Of course sensitivity decreased and specificity in-

Table 2 *Sero-positive results at different ELISA absorbance breakpoints in 182 asymptomatic females attending an STD clinic.*

Absorbance breakpoint	Groups of patients		
	Asymptomatic GC (n=52)	GC-contact; negative GC culture (n=26)	No GC (contact); negative GC culture (n=104)
⩾0.85	38 (73)*	13 (50)	36 (35)
⩾1.00	34 (65)	12 (46)	30 (29)
⩾1.15	32 (62)	10 (38)	19 (18)
⩾1.30	29 (56)	10 (38)	14 (13)

* percentage of positive sera in brackets.

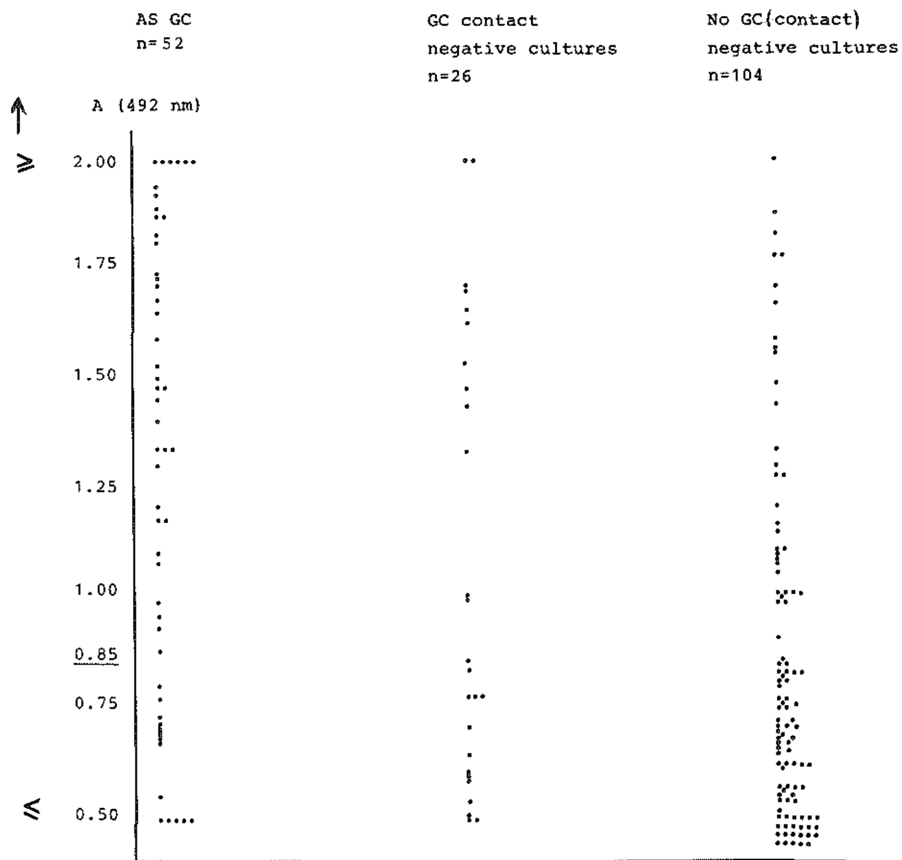

Fig 1
ELISA absorbance values in 182 asymptomatic females attending an STD clinic.

AS GC	=	patients with asymptomatic gonorrhoea
GC-contact	=	patients reporting as gonorrhoea contact with negative gonococcal cultures
No GC(contact)	=	patients not reporting as gonorrhoea contact and not suffering from gonorrhoea

creased as the breakpoint was raised (tables 2 and 3). A striking finding was that in the group of gonorrhoea contacts the number of positive sera did not diminish evenly, and that even at breakpoint A≥1.30 38% of these sera were still positive.

Table 3 *Sensitivity, specificity and predictive values of a positive [PV(+)] and of a negative [PV(−)] result at different ELISA absorbance breakpoints in 182 asymptomatic females attending an STD clinic*

Absorbance breakpoint	Sensitivity %	Specificity %*	PV(+)	PV(−)
≥0.85	73	62	0.44	0.85
≥1.00	65	68	0.45	0.83
≥1.15	62	78	0.52	0.83
≥1.30	56	82	0.55	0.82

* including gonorrhoea contacts.

The predictive value of a positive result was highest at A≥1.30 (PV(+)=0.55) but it exceeded 50% even at A≥1.15 (PV(+)=0.52). The predictive value of a negative result was highest at A≥0.85 (PV(−)=0.85).

Females without a history of gonorrhoea

Tables 4 and 5 present, in the same way as for the entire group of females, the results of the ELISA and their predictive values in asymptomatic females without a history of gonorrhoea. Comparison of tables 3 and 5 shows that the sensitivity is not influenced by previous gonococcal infections. The specificity increases slightly when females with a history of gonorrhoea are excluded.

Table 5 shows that the predictive value of a positive result is highest at A≥1.15 (PV(+)=0.57), and that of a negative result at A≥0.85 (PV(−)=0.87).

Table 4 *Sero-positive results at different ELISA absorbance breakpoints in 146 asymptomatic females without a history of gonorrhoea attending an STD clinic*

	Groups of patients		
Absorbance breakpoint	Asymptomatic GC (n=42)	GC-contact; negative GC culture (n=18)	No GC (contact); negative GC culture (n=86)
≥0.85	31 (74)*	9 (50)	23 (27)
≥1.00	28 (67)	9 (50)	18 (21)
≥1.15	26 (62)	9 (50)	11 (13)
≥1.30	20 (48)	9 (50)	9 (10)

* percentage of positive sera in brackets.

Table 5 *Sensitivity, specificity and predictive values of a positive [PV(+)] and of a negative [PV(−)] result at different ELISA absorbance breakpoints in 146 asymptomatic females without a history of gonorrhoea attending an STD clinic*

Absorbance breakpoint	Sensitivity %	Specificity %*	PV(+)	PV(−)
⩾0.85	74	69	0.49	0.87
⩾1.00	67	74	0.51	0.85
⩾1.15	62	81	0.57	0.84
⩾1.30	48	83	0.53	0.80

* including gonorrhoea contacts.

Discussion

Serological tests using gonococcal pili as antigen are highly sensitive in females with asymptomatic gonorrhoea. Buchanan et al.[1] attained a sensitivity of 89% with the RIA, and Oates et al.[2] attained 85% with the same test.

The incidence of asymptomatic infections in females with uncomplicated gonorrhoea attending an STD clinic is about 40-60%.[9]

The aim of this study was to establish what the value of the ELISA 6650 would be in the diagnosis of gonorrhoea in asymptomatic females if various absorbance breakpoints were considered in the test. Four breakpoints were arbitrarily chosen. With the aid of sensitivity, specificity and prevalence figures, the corresponding predictive values for a positive and for a negative result (PV(+) and PV(−)) were calculated. The best PV(+) was attained at A⩾1.15 and A⩾1.30, and the best PV(−) at A⩾0.85.

Of the patients studied, 20% had no history of gonorrhoea. When these were excluded, the PV(+) rose only at A⩾1.15 (not at A⩾1.30) and the PV(−) rose slightly.

As previously demonstrated (chapter 6) and illustrated in this paper, previously acquired gonorrhoea influences especially the specificity of tests using pili, and exerts a less marked influence on sensitivity, even if the previous gonorrhoea was acquired years ago.

There was a difference in specificity between patients reporting as gonorrhoea contact and the other patients with negative cultures. Young and Low[10], also using an ELISA with gonococcal pili as antigen, likewise found more positive test results in females reporting as gonorrhoea contact than in the other females with negative cultures. Among females reporting as gonorrhoea contact, there were undoubtedly a few culture failures. These patients were often treated on basis of the history, and in many cases, therefore, only one culture was made before treatment.

In a retrospective study of 210 females with gonorrhoea (cultured only from cervix and urethra), Evans[11] found that the diagnosis was missed at the first

examination in 10%. Moreover, at least a few percents of the gonococcal strains do not grow on the selective nutrient medium used here.[7] And finally, false negative results may be obtained due to irrigation of the vagina with bacteriolytic or bactericidal solutions and due to unreported use of antibiotics.

It can be stated in summary that, for use in asymptomatic females attending an STD clinic, the ELISA can be interpreted as follows. The result is positive at $A \geqslant 1.15$, and negative at $A < 0.85$, at which absorbances $PV(+)=0.52$ and $PV(-)=0.85$. When results are between $A=0.85$ and $A=1.15$, a new blood sample should be taken and the test repeated.

This version of the ELISA provides some help in the diagnosis of gonorrhoea in asymptomatic females attending an STD clinic.

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CHAPTER 10

General Discussion

Gonorrhoea is a sexually transmitted disease with a brief period of incubation. Females with asymptomatic infections are considered to be an important source of the spread of gonorrhoea. The development of a sensitive, specific serological test could be an important aid in the detection of gonorrhoea in this group and in other groups of patients. In this respect the GCFT using an impure antigen has not been satisfactory.

In recent years attempts have been made to enhance the sensitivity and specificity of tests for detection of gonorrhoea by using purified antigens such as pili[1-3], outer membrane protein[4] and, very recently, ribosomal fractions.[5] Highly sensitive test systems such as RIA[2], ELISA[1,4] and IHA[3,5] have been applied.

In this study pili of various gonococcal strains were prepared and purified for use as antigens. Paired sera and corresponding gonococcal strains were collected from patients with gonorrhoea attending an STD clinic, and sera were collected also from patients without gonorrhoea. Using pili prepared from strain 1443 as antigen, the value of taking paired sera was studied with the aid of an ELISA (ELISA 1443). It was found that, in males and females with uncomplicated gonorrhoea, the sensitivity of the assay rises when paired sera are tested. A relatively high sensitivity (61%) was attained in females with asymptomatic infections, but in this group the testing of the second serum did not further enhance sensitivity.

Next, the ELISA 1443 was compared with the IHA 2686 and with the GCFT routinely used in The Netherlands, with whole gonococci of a single strain (Oliver) as antigen. The pili in the IHA 2686 were prepared from an American strain originally used by Buchanan et al.[2] The ELISA 1443 and the IHA 2686 hardly differed in sensitivity and specificity. The sensitivity of the three tests was highest in oropharyngeal gonorrhoea and gonorrhoeal complications. The two assays using pili were much more sensitive than the GCFT. The specificity of all tests was very high in low-risk groups; in high-risk groups, however, the GCFT seemed more specific. The specificity of the pilus assays was influenced somewhat unfavourably by previous gonococcal infections.

The antigenic heterogeneity of gonococcal pili in human sera was studied with the aid of IHA inhibition tests. For this purpose, purified pilus preparations were prepared from gonococci isolated from patients. The sera from these patients were tested with antigen of the homologous strain as well as with pili of strain 2686. Among 22 pilus preparations examined, pili of two strains were found to differ in antigenic properties from each other as well as from pili of strain 2686. An important finding was that, with pili of strain 6650 (one of these preparations), the IHA attained a significantly higher sensitivity in

females with uncomplicated gonorrhoea (71%) than could be attained with pili of strain 2686. The sensitivity in the subgroup of females with asymptomatic infections was even as high as 75%. In the ELISA, too, pili of strain 6650 (ELISA 6650) scored significantly higher than strain 1443 pili.

Finally, the diagnostic value of the ELISA 6650 was studied in asymptomatic females attending an STD clinic. A study was made of the influence of a shift in absorbance breakpoint on the sensitivity and specificity of the test. It was found that the ELISA 6650 could be of some help in the diagnosis of asymptomatic gonococcal infections in females. The sensitivity and specificity of this pilus assay, however, are still far from optimal.

It can be concluded that the use of pilus assays markedly improves the serological diagnosis of gonorrhoea if compared with the GCFT as routinely used in The Netherlands. Yet in their present form these tests are not very suitable as a diagnostic aid in STD clinic populations. Their sensitivity is insufficient and their specificity too low. However, the pilus assays are suitable as a diagnostic aid in examining patients suspected of complicated gonococcal infections. Unfortunately, the specificity of the tests is insufficient in these cases, too, due to persistence of antibodies to gonococcal pili after previous gonococcal infections. The sensitivity and specificity of pilus assays might be improved by selection of pilus preparations of broader antigenic composition. This, however, should not lead to cross-reactions with meningococci or other *Neisseria* species.

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Summary

In The Netherlands, the gonococcal complement fixation test (GCFT) is commonly used in the serological diagnosis of gonorrhoea. This test is valuable in complicated forms of gonorrhoea but in uncomplicated forms it is unsatisfactory due to insufficient specificity and in particular insufficient sensitivity. The GCFT uses a crude antigen prepared from a particular strain by extraction.

In an effort to improve serodiagnosis, this thesis studies the way in which, in a gonococcal infection, antibodies are produced to a particular antigen at the surface of gonococci, i.e. pili. For this purpose gonococcal pili were purified and used in highly sensitive serological systems such as the enzyme-linked immunosorbent assay (ELISA) and the indirect haemagglutination assay (IHA). Paired sera and corresponding gonococcal strains were collected from patients with several forms of gonorrhoea; paired sera were also obtained from patients without gonorrhoea. On the basis of history, sex and diagnosis, the patients were divided into well-defined groups. Sera obtained on the day of the first examination (D1) and sera generally obtained 11-22 days later (D2) were examined.

Chapter 1 presents a brief historical review of the discovery of *Neisseria gonorrhoeae* and a general introduction which discusses the identification, cellular wall composition and typing of the bacterium.

Chapter 2 presents a survey of the literature on serological tests used to detect gonorrhoea and discusses the principal problems in the development of serological tests, e.g. gonococcal heterogeneity, persistence of antibodies from previous gonococcal infections, and cross-reactions with other *Neisseria* species.

Chapter 3 describes the aims and the design of the study presented in this thesis.

Chapter 4 discusses the techniques used to purify pili and describes the procedure of the ELISA.

Chapter 5 discusses the value of testing paired sera from patients with and without gonorrhoea by means of the ELISA with pili 1443 as antigen (ELISA 1443). In the groups of males and females with uncomplicated gonorrhoea, the use of a second serum sample distinctly enhanced the sensitivity of the test. In the group of females with asymptomatic infections it did not. The choice of the absorbance breakpoint in the test is discussed in an appendix to this chapter.

Chapter 6 describes a comparative study of the ELISA 1443, the GCFT routinely used in The Netherlands, and the IHA with pili 2686 as antigen (IHA 2686) in patients with and without gonorrhoea. In females with uncomplicated gonorrhoea the maximum sensitivity was 53% with the ELISA 1443, 58% with the IHA 2686 and 22% with the GCFT. The corresponding figures in

males with uncomplicated gonorrhoea were 41%, 50% and 14%, respectively. The three tests showed a high specificity in low-risk groups; in high-risk groups the specificity of the GCFT seemed to be higher.

Chapter 7 demonstrates the antigenic heterogeneity of gonococcal pili using human sera in the test system on the basis of indirect haemagglutination inhibition studies. With pili 6650 as antigen in the IHA (IHA 6650), a significant increase in sensitivity was observed in females with uncomplicated gonorrhoea if compared with the IHA 2686. In females with asymptomatic uncomplicated gonorrhoea the maximum sensitivity in the IHA 6650 was 75%.

Chapter 8 compares gonococcal pili 1443 and 6650 as antigen in the ELISA system. In females with uncomplicated gonorrhoea the sensitivity of the ELISA 6650 significantly exceeded that of the ELISA 1443 and equalled that of the IHA 6650.

Chapter 9 discusses the diagnostic value of the ELISA 6650 in asymptomatic females with and without gonorrhoea. When several absorbance breakpoints are used in the system, the ELISA 6650 can be of some help in the diagnosis of gonorrhoea in this patient group. Thus interpreted, the predictive value of a positive result [PV(+)] is 0.52, and that of a negative result [PV(−)] is 0.85.

Chapter 10 presents a general discussion and the principal conclusions from the study described in this thesis. In their present form, pilus assays are not quite suitable for use in STD clinic populations. The sensitivity of these assays is high in oropharyngeal gonorrhoea and gonorrhoeal complications. They are an important diagnostic aid when gonorrhoeal complications are suspected. The search for new, better pilus antigens should take into account the broad antigenic composition and heterogeneity of gonococcal pili; it should also establish whether cross-reactions with other *Neisseria* species occur, and whether previous gonococcal infections exert an unfavourable influence on specificity.

In this respect it should be borne in mind that the manner in which (as in this study) the gonococci and corresponding sera are collected and used is of essential importance for rapid and reliable comparison of antigens.

Samenvatting

In Nederland wordt voor de serologische diagnostiek van gonorroe als regel de gonococcen complement fixatie test (GCFT) toegepast. Deze test is wel van waarde bij gecompliceerde vormen van gonorroe, maar bij ongecompliceerde vormen van deze infectie voldoet deze test niet door gebrek aan specificiteit en vooral ook door een gebrek aan sensitiviteit. In de GCFT wordt gebruik gemaakt van een ruw antigeen, dat door extractie wordt bereid uit een bepaalde stam.

Met het doel de serodiagnostiek te verbeteren, wordt in dit proefschrift onderzocht hoe bij een gonorroïsche infectie de antilichaamvorming verloopt tegen een bepaald antigeen aan het oppervlak van gonococcen n.l. pili. Daartoe werden gonococcen pili gezuiverd en in zeer gevoelige serologische systemen zoals de enzyme-linked immunosorbent assay (ELISA) en de indirecte hemagglutinatie reactie (IHA) toegepast. Van patienten met gonorroe werden gepaarde sera en bijbehorende gonococcenstammen verzameld. Ook werden sera verzameld van patienten zonder gonorroe. De patienten werden op grond van anamnese, geslacht en diagnose ingedeeld in goed gedefinieerde groepen. Sera afgenomen op datum van eerste onderzoek (D_1) en in het algemeen 11-22 dagen later (D_2) werden onderzocht.

In een algemene inleiding wordt naast een kort historisch overzicht van de ontdekking van *Neisseria gonorrhoeae,* ingegaan op identificatie, celwandsamenstelling en typering van de bacterie (hoofdstuk 1).

Vervolgens wordt een literatuuroverzicht betreffende serologische testen ter detectie van gonorroe gegeven (hoofdstuk 2). De voornaamste problemen bij de ontwikkeling van serologische testen zoals gonococcen heterogeniteit, persisteren van antilichamen t.g.v. vroegere gonococcen infecties en kruisreacties met andere *Neisseria* worden genoemd.

Na beschrijving van de doelstelling en opzet van de studie (hoofdstuk 3), worden in hoofdstuk 4 methoden besproken, die werden gebruikt bij de zuivering van pili en wordt de methodiek van de ELISA beschreven.

De waarde van het testen van gepaarde sera bij patienten met en zonder gonorroe m.b.v. de ELISA met pili 1443 als antigeen (ELISA 1443) wordt in hoofdstuk 5 beschreven. Bij de groepen vrouwen en mannen met ongecompliceerde gonorroe gaf het bepalen van een tweede gepaard serummonster een duidelijke toename van de sensitiviteit te zien. By asymptomatisch geïnfecteerde vrouwen gaf het testen van het tweede serummonster geen extra bijdrage in de sensitiviteit. In de appendix van dat hoofdstuk wordt ingegaan op de keuze van het breekpunt in de test.

Dan wordt een vergelijkend onderzoek tussen de ELISA 1443, de in Nederland routinematig in gebruik zijnde GCFT en de indirecte hemagglutinatie reactie met pili 2686 als antigeen (IHA 2686) beschreven bij patienten met en zonder gonorroe (hoofdstuk 6). De maximale sensitiviteit bij vrouwen met ongecompliceerde gonorroe bedroeg 53% bij de ELISA 1443, 58% in de IHA 2686 en 22% in de GCFT. Bij mannen met ongecompliceerde gonorroe was de sensitiviteit resp. 41% in de ELISA 1443, 50% in de IHA 2686 en 14% in de GCFT. Bij low-risk groepen hebben de drie testen een hoge specificiteit, bij high-risk groepen lijkt de specificiteit van de GCFT hoger te zijn.

Antigene heterogeniteit van gonococcen pili met gebruik van humane sera in het test systeem werd gedemonstreerd door middel van indirecte hemagglutinatie inhibitie studies (hoofdstuk 7). Met pili 6650 als antigeen in de IHA (IHA 6650) werd een significante verhoging van de sensitiviteit bereikt bij vrouwen met ongecompliceerde gonorroe t.o.v. die van de IHA 2686. Bij

vrouwen met asymptomatisch ongecompliceerde gonorroe bedroeg de sensitiviteit in de IHA 6650 maximaal 75%.

In hoofdstuk 8 worden gonococcen pili 1443 en 6650 als antigeen in het ELISA systeem met elkaar vergeleken. Bij vrouwen met ongecompliceerde gonorroe is de sensitiviteit van de ELISA 6650 significant hoger dan die van de ELISA 1443 en gelijk aan die van de IHA 6650.

De diagnostische bruikbaarheid van de ELISA 6650 bij asymptomatische vrouwen met en zonder gonorroe, wordt in hoofdstuk 9 beschreven. Met het toepassen van meerdere breekpunten in het testsysteem kan de ELISA 6650 van enig nut zijn bij de diagnostiek van gonorroe bij deze patientengroep. Op deze wijze geïnterpreteerd is de predictieve waarde van een positief resultaat PV(+)=0.52 en van een negatief resultaat PV(−)=0.85.

Hoofdstuk 10 bevat een algemene discussie, waarin de belangrijkste conclusies van het in dit proefschrift beschreven onderzoek staan vermeld. Pilusassays zijn in hun huidige vorm weinig geschikt voor toepassing bij geslachtsziektenpolikliniek populaties. De sensitiviteit van de testen is hoog bij oropharyngeale gonorroe en gonorroïsche complicaties. Bij verdenking op gonorroïsche complicaties zijn ze een belangrijk diagnostisch hulpmiddel. Bij het zoeken naar nieuwe en betere pilus antigenen dient men rekening te houden met de breedte van de antigene samenstelling en heterogeniteit van gonococcen pili; ook dient men na te gaan of er kruisreacties met andere *Neisseria* optreden en of er een nadelige invloed van vroeger doorgemaakte gonorroïsche infecties op de specificiteit is. Daarbij is de manier, waarop gonococcen en daarbij behorende sera, zoals in dit proefschrift, verzameld en gebruikt worden, van essentieel belang om op snelle en verantwoorde wijze antigenen met elkaar te vergelijken.

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Curriculum vitae

De schrijver van dit proefschrift werd in 1948 te 's-Gravenhage geboren. In deze stad behaalde hij in 1966 het HBS-B diploma aan het Stevin Lyceum. Vervolgens studeerde hij medicijnen aan de Medische Faculteit Rotterdam, alwaar hij in 1973 het artsexamen aflegde.

Na het vervullen van de militaire dienstplicht begon hij in 1974 aan zijn specialisatie tot dermatovenereoloog op de afdeling Dermatologie en Venereologie (Hoofd: Prof. Dr. C. H. Beek, later waarnemend Hoofd Prof. Dr. E. Stolz) van het Academisch Ziekenhuis Dijkzigt Rotterdam. Gedurende één jaar (1975/1976) onderbrak hij zijn opleiding om het in dit proefschrift beschreven onderzoek te starten op de afdeling Klinische Microbiologie en Antimicrobiële Therapie (Hoofd: Prof. Dr. M. F. Michel).

Vanaf 1979 is hij verbonden als staflid aan de afdeling Dermatologie en Venereologie van het Academisch Ziekenhuis Dijkzigt Rotterdam en werkzaam als kinderdermatoloog in het Sophia Kinder Ziekenhuis Rotterdam.