

Development and evaluation of scheme for serotyping *Gardnerella vaginalis*

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SUMMARY Antibodies to *Gardnerella vaginalis* were raised in rabbits. Nine antisera that reacted with their immunising strains, but not with the remaining eight strains, were used to develop a serotyping scheme. A dot blotting technique was used, and complexes of antigen and antibody were visualised using anti-rabbit immunoglobulin linked to alkaline phosphatase. Of 91 clinical isolates used to evaluate the scheme, 79 (87%) were typable and 52 (57%) reacted with only a single antiserum. The antigens expressed were stable during growth on different media and on subculture. The specificity of the antibody was shown to be directed against different immunodominant proteins and possibly a carbohydrate.

Introduction

Though there is a close association between *Gardnerella vaginalis* and bacterial vaginosis,^{1,2} the epidemiology and pathogenic importance of *G vaginalis* in this condition remain poorly understood. A major problem in examining the role of *G vaginalis* in both vaginal and extrvaginal infection has been the lack of an effective reproducible typing system with good discrimination.

Piot *et al* have described a biotyping system for *G vaginalis*,³ but 80% of all strains tested belonged to three of the nine biotypes. Serotyping has also been attempted, but the system used was unsatisfactory.⁴

In the report published here we present the development and preliminary evaluation of a serotyping scheme using a dot immunobinding assay^{5,6} that has provided a rapid, convenient, and sensitive system for screening isolates. This procedure should discriminate isolates sufficiently to permit epidemiological studies.

Patients, materials, and methods

PREPARATION OF ANTISERA

Choice of strains

All strains of *G vaginalis* used to produce antisera were isolated from clinical samples from either women with bacterial vaginosis or their male contacts. They were identified by the methods described by Piot *et al*.⁷ Initially strains were chosen at random, but in later experiments strains that did not type with available antisera were used.

Antigens

Strains of *G vaginalis* were grown for 24 hours at 37°C in 5% carbon dioxide on 5% human columbia blood agar and then inoculated into a biphasic medium containing peptone starch dextrose agar⁸ and thioglycollate broth (Difco). After incubation for six hours on a rotary shaker at 37°C, the growth was harvested by centrifugation and the pellet resuspended in 1% formol saline and stored at 4°C for at least 48 hours. Before rabbits were immunised a small aliquot of the suspension was washed three times in phosphate buffered saline (pH 7.4) and the optical density adjusted to 0.1 (λ 540 nms).

Immunisation

New Zealand White rabbits weighing about 2 kg were inoculated using the following schedule: on day 1 0.5 ml bacterial suspension mixed at a ratio of 1:1 with

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incomplete Freund's adjuvant (Difco) was injected subcutaneously into 10 sites on the back; on day 7 1 ml bacteria and incomplete Freund's adjuvant was injected intramuscularly into both thighs; and on day 21 1 ml bacterial suspension alone was injected intravenously into the ear. All animals were test bled at day 28. Immunisation of some animals was boosted by a second intravenous injection before they were bled.

CHOICE OF ANTISERA

Antisera were titrated against their own immunising strain and other strains of *G vaginalis* used to produce antisera, using a dot blotting technique. We chose nine antisera that were active against their immunising strain only and showed no activity against the remaining eight strains.

SEROTYPING

Strains

We chose 91 isolates of *G vaginalis* to evaluate the technique. All strains were isolated from the vaginas of women with bacterial vaginosis on 5% human bilayer agar (HBA) plates containing gentamicin, nalidixic acid, and amphotericin.⁹ Each strain was subcultured from a single colony to avoid the presence of more than one serotype per clinical specimen, and was identified as *G vaginalis* using the methods of Piot *et al.*⁷ All bacteria were stored at -70°C in 10% glycerol broth between experiments.

Preparation of whole cell antigens

G vaginalis was grown on HBA for 48 hours in 5% carbon dioxide. A suspension of bacteria was prepared in saline, and the optical density was adjusted to 0.05 (λ 540 nm).

Dot blotting technique

A nitrocellulose membrane (Anderman) measuring 12 × 8 cm was soaked in saline for 10 minutes and placed in a Bio-Dot blotter (Bio-Rad Laboratories). A volume of 100 μ l bacterial suspension was added to each well and left at room temperature for 30 minutes. The bacteria were then concentrated on the membrane by the application of a vacuum. The membrane was soaked in blocking buffer (4% bovine serum albumin (Sigma) and 0.5% polysorbate (Tween) 20 (Sigma) in enzyme linked immunosorbent assay (ELISA) buffer (0.15 mol/l sodium chloride and 10 mmol/l TRIS) (pH 7.4)) on a rotary shaker for 30 minutes at 37°C. The membrane was then immersed in antiserum diluted either 1:5000 or 1:10 000 in 0.5% bovine serum albumin in ELISA buffer (BSA buffer) and incubated overnight at 37°C on a shaker. The membrane was rinsed in saline and then washed in BSA buffer three times for 30 minutes each time. Antirabbit immunoglobulin linked to alkaline

phosphatase (Dakopatts) diluted 1:5000 in BSA buffer was then added, and incubated for four hours at 37°C. The complexes of antigen and antibody were visualised by washing the membrane as previously described and then immersing it in substrate (0.75 ml 5-bromo-4-chloro-3-indolyphosphate (Sigma) 4 mg/ml in a mixture of methanol and acetone at a ratio of 2:1 (v/v); 5 ml nitroblue tetrazolium (Sigma) 1 mg/ml; 0.2 ml magnesium chloride 1 mol/l; 44.05 ml ethanolamine - hydrochloric acid buffer 0.1 mol/l (pH 9.6)) for 10 minutes at room temperature. The reaction was terminated by washing the membrane in distilled water, blotting, and air drying.

Reading

All nine immunising strains were placed on each membrane carrying test strains, to check the specificity of antiserum and to aid the interpretation of the results. Each membrane was read macroscopically by three observers and scored as follows: 0 (no reaction), 1 (weak reaction of less intensity than that of the control), or 2 (strong reaction of equal intensity to that of the control strain).

Stability of antigens

To assess the effect on the serotype the nine immunising strains and 11 clinical isolates were inoculated on to the following media: horse and human blood agar (with or without antibiotics), peptone starch dextrose agar and broth, or thioglycollate broth or fastidious anaerobe broth, both with 10% horse serum added. Bacteria were harvested from broth culture by centrifugation and washed twice in saline; suspensions were prepared for dot blotting as above.

SEROTYPE DISTRIBUTION AND STABILITY IN CLINICAL ISOLATES

The number of serotypes present in a primary isolate of *G vaginalis* was tested using vaginal swabs from women with "clue" cells in their vaginal discharge. The swabs were cultured on selective human blood agar for 48 hours, and 10 haemolytic colonies were then subcultured separately on to non-selective HBA. The growth after 48 hours was used for serotyping and full identification.

The effect of continued subculturing was assessed using single colony subcultures both from fresh isolates and strains stored at -70°C.

BIOTYPING

All 91 strains of *G vaginalis* used to evaluate the serotyping scheme were also biotyped using the methods described by Piot *et al.*³

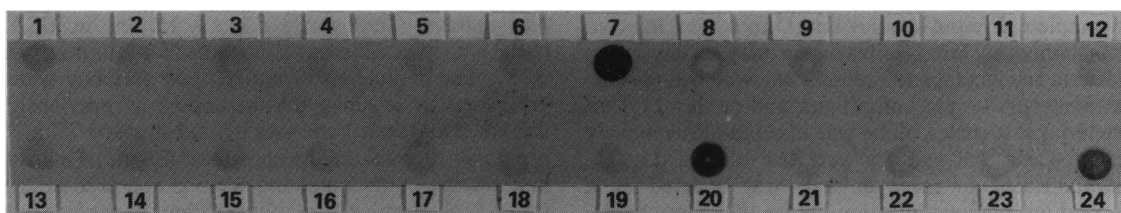


FIG 1 Example of dot blot test with antiserum H. Positive reactions at spots 7, 20, and 24. Spots 1-9 = immunising strains, 11 = *Lactobacillus acidophilus*, 12-24 = clinical isolates.

IMMUNOBLOTTING

Each immunising strain of *G vaginalis* was grown for 48 hours on HBA, harvested, and washed in saline. The protein concentration was estimated¹⁰ and adjusted to 2 mg/ml. The suspension was diluted with an equal volume of double strength sampling buffer (0.1% bromophenol blue, 2.0% sodium dodecyl sulphate, 8% v/v TRIS-hydrochloric acid 1 mol/l (pH 6.8), and 10% glycerol) containing 0.1 mol/l Clelands reagent, and was boiled for 10 minutes.

The whole cell lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Lammeli¹¹ using a 10% separating gel with a 5% stacking gel. Samples containing 5 µg protein were placed on the gel together with protein standards (low molecular weight standards, Bio-Rad Laboratories). The dye front was run 7-8 cm into the separating gel, and the proteins were then transferred electrophoretically to a nitrocellulose membrane using a semidry electroblotter (Ancos, Denmark) incorporating anodic buffer No 1 (0.3 mol/l TRIS, 20% methanol, and 80% distilled water), anodic buffer No 2 (25 mmol/l TRIS, 20% methanol, and 80% distilled water), and cathodic buffer (25 mmol/l TRIS, 40 mmol/l 6-amino-n-hexanoic acid, 20% methanol, and 80% distilled water).¹² The gels were blotted for two hours at 220 mA.

The membrane was then either stained for protein using 0.1% amido black in 25% methanol and 10%

acetic acid or it was placed in blocking buffer (as for dot blotting). Each antiserum was tested at a dilution of 1:10 000 against its immunising strain using the method described above.

Results

Dot blotting was chosen because it permitted many strains to be tested against a variety of antisera. The blue colour produced by the alkaline phosphatase gave a result that was easy to read (fig 1), though different degrees of colour did occur. We devised a scoring system to assess and overcome differences in the interpretation of results by different observers. A score of zero indicated a negative result, a weaker reaction than that of the immunising strain was scored as 1, and a reaction equal to or greater than that of the immunising strain was scored as 2. The scores from all three observers were summed, and a score of 6 indicated that all observers had assessed the result as being strongly positive whereas a score of 1 indicated that a weak reaction had been noted by only one observer. Table I shows the effect of different scores on the number of strains that were typable and the number of strains that reacted with only a single antiserum. It also shows the total number of serotypes. A score of 3 showed that each observer had found that the strain gave at least a weak reaction. We thought that this score gave acceptable typability (79/91, 87%) and the largest number of

TABLE I Selection of criteria for evaluating serotyping

Sum of scores	Minimum individual scores* by observers:			No (%) of isolates typed (n=91)	No (%) of isolates reactive with single antiserum	Total no of serogroups
	1	2	3			
6	2	2	39 (43)	34 (37)	12	5
2	2	1	57 (63)	49 (54)	14	4
1	1	2	65 (71)	50 (55)	16	3
1	1	1	79 (87)	52 (57)	20	2
1	1	0	84 (92)	45 (50)	28	

*0 = negative, 1 = weak reaction (less than that of control), 2 = strong reaction (equal or greater than that of control). Antibody dilution = 1:10 000.

TABLE II Serotypes of 91 strains of *Gardnerella vaginalis*

Serotype	No (%) of strains	Serotype	No (%) of strains
A	4 (4)	CF	4 (5)
B	2 (2)	DF	2 (2)
C	23 (25)	ACF	3 (3)
D	8 (9)	ADF	1 (1)
E	3 (3)	CDF	1 (1)
F	7 (8)	CHJ	1 (1)
G	3 (3)	ACDF	1 (1)
H	2 (2)	ABCDF	3 (3)
J	2 (2)	ABDFH	1 (1)
AD	1 (1)	Non typable	12 (13)
AF	7 (8)		

strains reacting with a single antiserum (52/91, 57%). The 20 resulting serotypes gave sufficient discrimination. Though there was little difference between antibody dilutions of 1:5000 and 1:10 000, the latter was chosen for routine use.

Table II shows the distribution of serotypes, serotype C being the most common. The reproducibility of the serotype of strains reacting with one antiserum was 100%. Mixed serotypes showed good reproducibility, though a weak reaction was occasionally lost.

The antigens detected by these polyclonal antibodies were also expressed after growth on horse and human blood agar (with or without antibiotics) or peptone starch dextrose broth and agar. After growth in thioglycollate broth or fastidious anaerobe broth the reactions were weak and difficult to detect, though there was no change in serotype. The expression of antigens was stable on continued subculture. We were unable to detect the presence of more than one serotype in 10 primary isolates of *G vaginalis*, though occasional non-reactive colonies have been encountered. Single colony subculture of strains previously subcultured from one colony and of known serotype also showed some non-reactive colonies.

The distribution of biotypes in our strains was biotype 1 (13, 14%), 2 (31, 34%), 3 (4, 4%), 4 (5,

TABLE III Comparison of biotype and serotype of 68 strains of *Gardnerella vaginalis*

Serotype	No of strains of biotype:							
	1	2	3	4	5	6	7	8
A	1	2			1			
B				2				
C		14	1		8			
D	1	3	1	1	1		1	
E							3	
F	2	1			4			
G							3	
H		2						
J		1			1			
AF	1				5		1	
CF		2			2			
ACF		3						

TABLE IV Specificity of antisera raised to *Gardnerella vaginalis*

Antisera	Immunodominant bands:	
	Protein (molecular weight in kilodaltons)	Carbohydrate
A	45	Present
B	44, 56	Absent
C	48, 94	Absent
D	46	Present
E	40, 54	Absent
F	45	Present
G	46, 56	Absent
H	37, 41, 90	Present
J	47	Present

6%), 5 (27, 30%), 6 (0), 7 (9, 10%), and 8 (2, 2%). The reproducibility of biotyping was good if a heavy inoculum was used for hippurate hydrolysis. Table III compares the biotypes with the main serotypes. It will be difficult to show correlation between biotype and serotype until a larger number of strains have been tested.

The specificity of the antibody raised to *G vaginalis* was largely directed to proteins of differing molecular weights (table IV). Five antisera also contained antibody directed to a substance that runs just before the dye front on SDS-PAGE. Staining of the gel with periodic acid Schiff produced a red colour indicative of a carbohydrate at the same position on the gel. Examples of the immunoblots, showing the presence of immunodominant lines, are shown in figure 2 using antisera at a dilution of 1:10 000. Some of these immunodominant bands occurred at the same molecular weight, but it was not possible using this technique to establish whether they possessed the same epitope. At lower dilutions the presence of cross reactive bands could be detected.

Discussion

Despite considerable work in the past 30 years, the pathogenicity and epidemiology of *G vaginalis* remains controversial and confused. Previous attempts at serological classification have not been exploited. We have found that it is relatively easy to produce antibody to *G vaginalis* in rabbits and to obtain individual reagents that show suitable discrimination for developing a serotyping scheme without the need for absorption. The choice of immunising strains was crucial to a good typing scheme. The ideal strain would produce an antiserum that gave clear differentiation between positive and negative results. In our panel of nine strains we have produced some such antisera. Some of the antisera, however, detected antigens expressed weakly in a large number of strains, and hence resulted in many weak reactions. It is our aim to replace these with new antisera showing a greater specificity. This may be

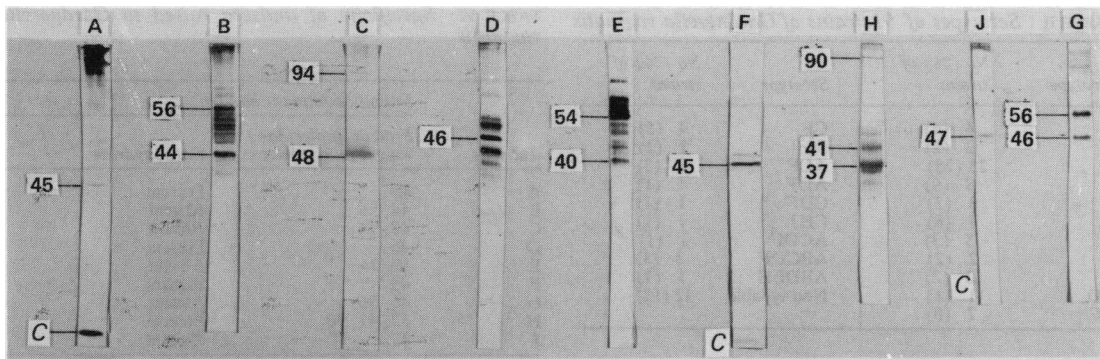


FIG 2 Immunoblots showing characteristic dominant lines of immunising strains. (Figures denote molecular weight of major bands in kilodaltons. C = reaction with possible carbohydrate).

achieved by using different strains to produce antisera in rabbits. It may, however, be more profitable to produce polyclonal antibody to previously identified single immunodominant bands cut or eluted from SDS-PAGE. An alternative would be the use of monoclonal antibodies.

The choice of a detection system was determined by sensitivity and ease of handling a large number of strains. Immunofluorescence has been the method chosen for many new immunodiagnostic techniques, but it requires skill and experience by the reader and is unsuitable for large numbers of tests. The dot blotting enzyme linked assay we have developed has long incubation and washing times. This is not as great a disadvantage for a typing scheme as for a detection test. All enzyme linked assays, however, can be manipulated to produce a faster system by changing the dilutions of antibody and conjugates used. An ELISA performed in microtitre trays, which would give a quantitative result, might overcome problems in interpretation, but we think that this would be less acceptable to routine clinical laboratories.

The serotyping scheme described has shown that it will be possible to produce an epidemiological tool for typing *G vaginalis*. The present panel of antisera is not ideal, but it shows the potential of *G vaginalis* to present different immunodominant proteins. We think that the present system will permit preliminary epidemiological studies while new more specific antibodies are produced. Several epidemiological questions could be approached. The prevalence of *G vaginalis* in the urethra and urine of male contacts of women with bacterial vaginosis has been shown to be high.^{13 14} It has been impossible, however, to confirm the association without a suitable typing scheme. Differences between isolates of *G vaginalis* from women with and without the characteristic signs of bacterial vaginosis² could also be studied.

The biotyping scheme described by Piot *et al*³ has not been used extensively for epidemiological studies.

In our hands the scheme was reproducible if a heavy inoculum was used for the hippurate hydrolysis, to overcome the inoculum effect. The major drawback is the distribution of biotypes. In the original study 80% of strains were of biotypes 1, 2, or 5. We found that 78% (71/91) of our isolates also belonged to these biotypes. A recent modification of the biotyping scheme includes the fermentation of arabinose, galactose, and xylose.¹⁵ This scheme identified 17 biotypes in 197 strains, but the distribution of biotypes was similar in isolates from asymptomatic and symptomatic women.

A combination of biotyping and serotyping could possibly be used to aid discrimination, particularly in major serotypes such as serotype C.

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