JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1984, p. 255–258
0095-1137/84/080255-04$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 20, No. 2

Filter Radioimmunoassay, a Method for Large-Scale Serotyping of *Neisseria meningitidis*

SIMON DE MARIE,† JAN H. J. HOEIJMAKERS, JAN T. POOLMAN, AND H. C. ZANEN*

Netherlands Reference Laboratory for Bacterial Meningitis, Department of Medical Microbiology, University of Amsterdam, Academisch Medisch Centrum, 1105 AZ Amsterdam, The Netherlands

Received 30 November 1983/Accepted 18 April 1984

A simple and rapid filter radioimmunoassay method can be used to serotype meningococcal strains on a large scale. The technique consists of simultaneous inoculation of 96 strains on nitrocellulose filters. The resulting colonies can be processed in situ, by extraction and fixation, incubation with antibodies and ^{125}I-labeled protein A, and, finally, autoradiography. Processing many filters simultaneously, one person can serotype thousands of meningococci in a week. Multiple filters with identical strain patterns can be stored after the fixation step for future screening. The use of monoclonal antibodies is essential; polyclonal antisera, even after extensive absorption, were not specific in this assay. When results from filter radioimmunoassay and Ouchterlony microprecipitation were compared for the serotyping of 201 *Neisseria meningitidis* strains for serotypes 2a and 2b, filter radioimmunoassay was sufficiently sensitive and specific to be useful in mass screening.

In addition to serogrouping on the basis of capsular polysaccharides, serotyping based on outer membrane proteins (OMPs) supplies useful information about the spread of virulent meningococcal strains (7, 9). Among the 15 serotypes described by Frasch (4), serotype 2 has been found frequently among isolates from patients with meningococcal disease but infrequently among isolates from carriers, indicating an association with virulence (1, 3, 4, 7, 9, 11, 12, 16). Serotype 2 has been subdivided into three subtypes: 2a, 2b, and 2c, which appear to have different distributions among the serogroups (16).

Current serotyping systems are complicated by the involvement of different molecules (different OMPs, lipopolysaccharides), some of which are highly variable (13, 15, 17, 19). To overcome these problems, Frasch et al. recently proposed a new nomenclature for designation of serotypes (C. E. Frasch, W. D. Zollinger, and J. T. Poolman, submitted for publication) based on the recognition of five classes of OMPs which differ in molecular weight and in degree of variability. The principal protein serotypes, including classes 2 and 3 OMPs (molecular weights, 37,000 to 39,000 and 40,000 to 42,000, respectively), that are stably expressed by the meningococcus are those most suitable for use in epidemiological studies. To conform with this classification, serotyping results obtained in the past must be reevaluated to confirm the typing and to permit comparison with other studies. A method for large-scale serotyping, using well-characterized antisera, is very helpful—not only for retrospective screening of collections of meningococci for the presence of the now well-characterized serotypes, but also for establishing the occurrence of newly identified serotypes in the future.

Most of the serotyping methods in common use are laborious and not suitable for testing large numbers of strains (2, 5, 6, 8, 12, 19). Therefore, we have adapted and modified the radioimmunological screening method developed by Henning et al. (10) so as to apply it in mass serotyping. This filter radioimmunoassay (FRIA) was used with monoclonal antibodies directed against serotypes 2a and 2b to test 201 *Neisseria meningitidis* strains, and the results were compared with those obtained from immunoprecipitation (16).

(A preliminary report of the FRIA technique has been published as an abstract [S. de Marie, J. H. J. Hoeijmakers, J. T. Poolman, and H. C. Zanen, Antonie van Leeuwenhoek J. Microbiol. Serol. **47**:470–472].)

MATERIALS AND METHODS

Strains. All strains of *N. meningitidis* came from the collection of the Netherlands Reference Laboratory for Bacterial Meningitis (comprising nearly 3,500 meningococcal strains collected since 1959) and had been stored at −70°C in a peptone broth containing 15% glycerol. A total of 201 strains, located between 1966 and 1978 from 167 patients with systemic meningococcal infection and from 34 asymptomatic carriers, were used to determine the reliability of serotyping by the FRIA. The 201 strains were selected on the basis of availability of Ouchterlony immunoprecipitation data from earlier studies (15, 16), which would allow comparison with FRIA for serotyping. Serogrouping by agglutination and microprecipitation was performed as previously described (16). These 201 well-characterized strains were not handled separately, but were included in the screening of the entire collection of meningococcal strains so that we could assess the usefulness of the FRIA typing as a large-scale procedure. In this way, each run of 96 samples included some whose typing could then be checked against that obtained by Ouchterlony immunoprecipitation.

FRIA. (i) Filter cultures. Glycerol-peptone broth suspensions of strains were transferred to 96-well microtiter plates. With a multipoint inoculator (Denley A 400), these were then inoculated in the same pattern onto nitrocellulose filters (Millipore HAWP), which were placed on top of Mueller-Hinton agar plates supplemented with 1% (vol/vol) yeast extract. The filters were 8.5 by 12.5 cm; the agar plates were 14 by 14 cm. The sterile filters were placed for a few minutes on a fresh agar plate to wet them before inoculation. The cell suspensions in each microtiter well were sufficient to inoculate 20 filters; suspensions containing between 10^4 and 10^6 bacteria per ml resulted in adequate growth. The bacteria were allowed to grow on the filters for 6 to 10 h at 37°C in 5% CO_2. No differences were observed between growth on filters and on agar plates. The replica filters, containing

* Corresponding author.
† Present address: Department of Internal Medicine, University Hospital, Utrecht 3511 GV, The Netherlands.

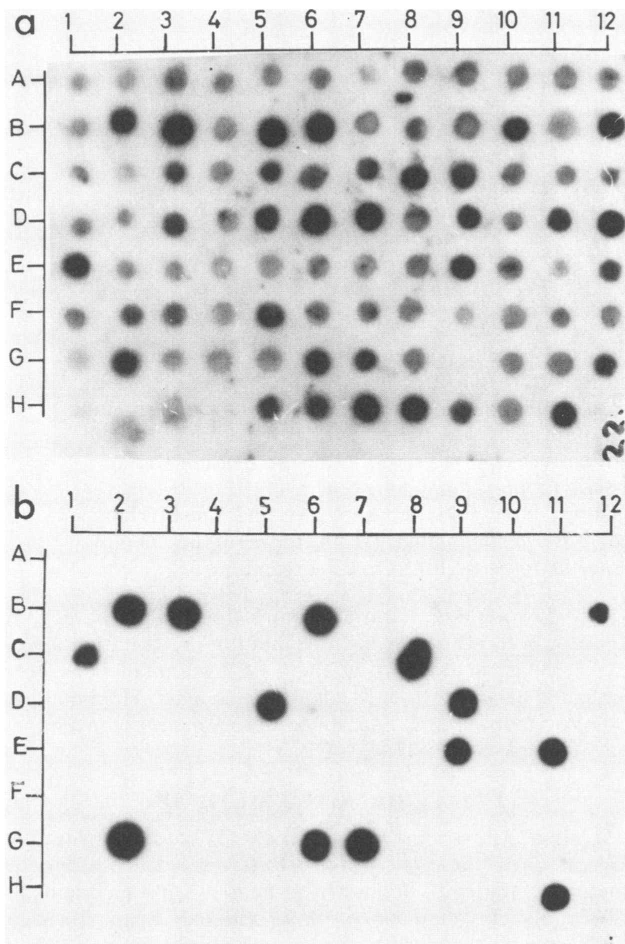

FIG. 1. FRIA, using polyclonal and monoclonal anti-2a. (a) Autoradiogram of one filter culture containing 96 meningococcal strains after incubation with polyclonal anti-2a. The positions of the strains are indicated by 1 through 12 horizontally and A through H vertically; (b) autoradiogram of an identical filter culture after monoclonal anti-2a.

clearly visible colonies in a pattern identical to that of the master microtiter plate, were then lifted from the agar and air dried.

Fixation of the bacteria on the filters and exposure of the serotype antigens were achieved by a 1-min incubation of the filters in a phenol-chloroform-heptane mixture (5:5:8 [vol/vol]), followed by a 15-min wash in chloroform-methanol (2:1). The filters were then air dried. At this stage, they could be stored at −20°C in a sealed box for several years without significant loss of antigenic activity.

(ii) Radioimmunoassay. To reduce nonspecific binding of antibody molecules, the filters were floated for 5 h at room temperature in phosphate-buffered saline (PBS) (140 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4 [pH 7.4]) containing 3% (wt/vol) bovine serum albumin (fr V, Sigma). The filters were drip dried and then were incubated with serotype-specific antibodies by laying each filter on a film of 0.8 ml of antibody solution (1:30 diluted absorbed polyclonal rabbit antiserum or a 1:100 dilution of mouse ascites fluid containing monoclonal antibodies) in a petri dish. The filters were placed one on top of another, special care being taken to avoid air bubbles between the filters. The rabbit antiserum was diluted with PBS (pH 7.4); the monoclonal antibody solution was diluted with PBS (pH 7.2) containing 0.5% (wt/vol) bovine serum albumin and 0.2% (vol/vol) Triton X-100. After overnight incubation at 4°C, unbound antibody molecules were removed by thorough washing in five changes of PBS supplemented with Nonidet P-40 and Tween 80 (both 0.8% [vol/vol]) over at least 4 h at room temperature.

After a second incubation with 3% bovine serum albumin in PBS for 1 h at room temperature, ^{125}I-labeled protein A of *Staphylococcus aureus* (labeled by the chloramine T method [14] to a specific activity of 10^8 dpm/μg/ was added and allowed to react with bound antibody molecules for 30 min at room temperature. Unbound protein A was removed by thorough washing in an excess of PBS containing Nonidet P-40 and Tween 80 (both 0.8%) for 20 h, on a shaker and with at least five changes of rinsing solution. The dried filters were exposed overnight to Kodak X-Omat R autoradiographic film at −70°C with an Ilford intensifying screen. Useful FRIA serotyping should result in clearly discernible spots of serotype-positive bacterial colonies on the X-ray plan, whereas negative colonies should be hardly visible.

Antisera. Polyclonal anti-2a and anti-2b rabbit antisera were absorbed thoroughly with heterologous serotype strains as previously described (16). These antisera were subsequently absorbed thoroughly by incubation with lysates of heterologous serotype strains to eliminate antibodies against cell wall or intracellular constituents. Lysates were obtained by sonication (using a Branson sonifier for 5 min at 70 W with 50% interruption for cooling) of a dense bacterial suspension. Absorbed antisera were recovered from the incubation mixture by centrifugation at 100,000 × *g* and 4°C for 20 min.

Monoclonal anti-2a and anti-2b immunoglobulin G antibodies were a generous gift of Wendell D. Zollinger (Walter Reed Army Institute of Research, Washington, D.C.). These monoclonal antibodies had been characterized by gel immunoradioassay (18) as specific for the class 2 proteins of the respective serotypes.

RESULTS

In FRIA, serotyping for the presence of serotypes 2a and 2b was performed by using two kinds of antibody reagents: absorbed polyclonal rabbit antisera and monoclonal antibodies. Figure 1 shows autoradiograms of a nitrocellulose filter after reaction with extensively absorbed polyclonal anti-2a and with monoclonal anti-2a. When absorbed polyclonal antisera were used, the FRIA yielded a spectrum of various densities without sufficient distinction between positive and negative colonies. In contrast, monoclonal antibodies gave clearly readable results. Monoclonal anti-2b showed somewhat higher background reactions as compared with monoclonal anti-2a, but the results were still easy to interpret.

To determine the sensitivity and specificity of the FRIA by using monoclonal antibodies, Ouchterlony microprecipitation was used as a reference based on the criterion of immunological identity. A total of 201 strains had been tested by Ouchterlony microprecipitation. These same strains were tested blind in the FRIA, distributed among the nearly 3,500 meningococcal strains being screened. The typing results were then extracted for comparison with the microprecipitation results for detection of both serotypes. The comparisons are summarized in Tables 1 and 2. FRIA failed to detect one serotype 2a strain that was positive in immunoprecipitation, but all of the other 200 strains were typed the same by both methods. Monoclonal anti-2b gave more discrepancies with immunoprecipitation (Table 2): 3 of 155 (1.9%) were false-positive, and 5 of 46 (10.9%) were

false-negative. Variability in growth did not influence the typing results. Just-visible growth appeared to be adequate for serotyping.

DISCUSSION

For a reference laboratory such as ours, the development of a serotyping technique that allows the screening of thousands of meningococci is desirable for evaluating the distribution of serotypes over periods of many years. The FRIA technique described in this article overcomes the problem of handling each strain individually, as is necessary in other serotyping methods (2, 5, 6, 8, 12, 19). Each stage of the FRIA procedure (growth, fixation, exposure of the serotype antigens, reaction with antibodies, and labeling of the bound antibodies) is done simultaneously for 96 strains on one nitrocellulose filter. By simultaneously processing many filters, it is indeed possible for one person to serotype thousands of meningococcal strains within a week. Because multiple filters with identical bacterial patterns can be stored after the fixation step, large collections of strains can be kept for future screening for the presence of yet unknown antigens as soon as monoclonal antibodies are available. In view of the recently proposed revision of classification (Frasch et al., submitted for publication), this is of special importance for retrospective study of newly defined serotypes.

The low degree of specificity displayed by extensively absorbed polyclonal antisera in FRIA might be due to exposure of cross-reactive immunodeterminants by the fixation and extraction procedure. Agglutination techniques may avert such undesirable cross-reactions since they leave the outer membrane of the bacteria intact; in the case of immunoprecipitation, isolated outer membranes are used as antigens. We concluded that both the anti-2a and the anti-2b polyclonal antisera employed in this study were inadequate for the FRIA method. However, this problem of nonspecificity was overcome by the use of monoclonal antibodies.

In this study, FRIA was compared with immunoprecipitation as a reference, using the criterion of immunological identity (16). The FRIA results with anti-2a were excellent (no false-positive and one false-negative reaction). The results with anti-2b showed higher failure rates (1.9% false-positive, 10.9% false-negative), but they were still acceptable for mass screening purposes. This might indicate that the immunodeterminant recognized by the monoclonal anti-2b had been modified by the FRIA procedure in a few cases.

TABLE 1. Comparison of meningococcal serotyping by FRIA and Ouchterlony immunoprecipitation for serotype 2a

Source of meningococci	Serogroup	No. of strains found to be: Anti-2a positive by immunoprecipitation: Positive by FRIA	Anti-2a positive by immunoprecipitation: Negative by FRIA	Anti-2a negative by immunoprecipitation: Positive by FRIA	Anti-2a negative by immunoprecipitation: Negative by FRIA	No. in serogroup
Patients with disease	A	0	0	0	20	20
	B	1	0	0	86	87
	C	10	0	0	22	32
	W-135	15	1	0	2	18
	Y	0	0	0	10	10
Carriers	B	2	0	0	8	10
	C	1	0	0	9	10
	W-135	4	0	0	2	6
	Y	0	0	0	8	8

TABLE 2. Comparison of meningococcal serotyping by FRIA and Ouchterlony immunoprecipitation for serotype 2b

Source of meningococci	Serotype	No. of strains found to be: Anti-2b positive by immunoprecipitation: Positive by FRIA	Anti-2b positive by immunoprecipitation: Negative by FRIA	Anti-2b negative by immunoprecipitation: Positive by FRIA	Anti-2b negative by immunoprecipitation: Negative by FRIA	No. in serogroup
Patients with disease	A	0	0	0	0	0
	B	40	5	2	40	87
	C	1	0	0	31	32
	W-135	0	0	1	17	18
	Y	0	0	0	10	10
Carriers	B	0	0	0	10	10
	C	0	0	0	10	10
	W-135	0	0	0	6	6
	Y	0	0	0	8	8

Perhaps other monoclonal antibodies against serotype 2b could be selected to give a high level of specificity and sensitivity, as did the anti-2a.

The discrepancies between the polyclonal antisera and monoclonal antibodies revealed in the FRIA again emphasize the need for characterization and standardization of the antisera used in serotyping.

In conclusion, the FRIA technique described in this article is a method very suitable for large-scale (OMP) serotyping of meningococci, using monoclonal antibodies. The method is relatively simple, reproducible, and economical with respect to antibodies, and yields specific and clearly visible results. The results for more than 3,000 meningococcal strains typed by monoclonal anti-2a and anti-2b will be described elsewhere (S. de Marie, J. T. Poolman, P. Bol, L. Spanjaard, and H. C. Zanen, submitted for publication).

ACKNOWLEDGMENTS

This work was supported in part by grants from the Praeventie Fonds (no. 28-310) and from Beecham Farma B.V. (no. BE-A79.003).

We are very grateful to Wendell D. Zollinger for providing the essential monoclonal antibodies. We are indebted to Carla T. P. Hopman, Pieter Bol, and Tineke Riemens for technical assistance and to Helena Ross and Pamela Wright for reviewing the manuscript.

LITERATURE CITED

1. **Ashton, F. E., A. Ryan, B. B. Diena, A. M. R. Mackenzie, and F. Chan.** 1980. Serotypes among *Neisseria meningitidis* serogroups B and C strains isolated in Canada. Can. J. Microbiol. **26**:1480–1488.
2. **Danielsson, D., and P. Olcén.** 1979. Rapid serotyping of groups A, B, and C meningococci by rocket-line immunoelectrophoresis and co-agglutination. J. Clin. Pathol. **32**:136–142.
3. **De Mayer, S., J.-M. Seba, and G. Reginster.** 1981. Epidemiology of meningococcal meningitis in Belgium. J. Infection **3**:63–70.
4. **Frasch, C. E.** 1979. Noncapsular surface antigens of *Neisseria meningitidis*. Semin. Infect. Dis. **2**:304–337.
5. **Frasch, C. E., and S. S. Chapman.** 1972. Classification of *Neisseria meningitidis* group B into distinct serotypes. II. Extraction of type-specific antigens for serotyping by precipitin techniques. Infect. Immun. **6**:127–133.
6. **Frasch, C. E., and S. S. Chapman.** 1972. Classification of *Neisseria meningitidis* group B into distinct serotypes. I. Serological typing by a microbactericidal method. Infect. Immun. **5**:98–102.
7. **Gold, R., J. L. Winklehake, R. S. Mars, and M. S. Artenstein.**

1971. Identification of an epidemic strain of group C *Neisseria meningitidis* by bactericidal serotyping. J. Infect. Dis. **124**:593–597.

8. **Gold, R., and F. A. Wyle.** 1970. New classification of *Neisseria meningitidis* by means of bactericidal reactions. Infect. Immun. **1**:479–484.
9. **Griffis, J. M., D. D. Broud, C. A. Silver, and M. S. Artenstein.** 1977. Immunoepidemiology of meningococcal disease in military recruits. I. A model for serogroup independency of epidemic potential as determined by serotyping. J. Infect. Dis. **136**:176–186.
10. **Henning, U., H. Schwarz, and R. Chen.** 1979. Radioimmunological screening method for specific membrane proteins. Anal. Biochem. **97**:153–157.
11. **Jacobson, J. A., T. J. Chester, and D. W. Fraser.** 1977. An epidemic of disease due to serogroup B *Neisseria meningitidis* in Alabama: report of an investigation and community-wide prophylaxis with a sulfonamide. J. Infect. Dis. **136**:104–108.
12. **Jones, D. M., and B. M. Tobin.** 1976. Serotypes of group B meningococci. J. Clin. Pathol. **29**:746–748.
13. **Mandrell, R. E., and W. D. Zollinger.** 1977. Lipopolysaccharide serotyping of *Neisseria meningitidis* hemagglutination inhibition. Infect. Immun. **16**:471–475.
14. **McConahey, P. J., and F. J. Dixon.** 1980. Radioiodination of proteins by the use of the chloramine-T method. Methods Enzymol. **70**:210–213.
15. **Poolman, J. T., S. de Marie, and H. C. Zanen.** 1980. Variability of low-molecular-weight, heat-modifiable outer membrane proteins of *Neisseria meningitidis*. Infect. Immun. **30**:642–648.
16. **Poolman, J. T., C. T. P. Hopman, and H. C. Zanen.** 1980. Immunochemical characterization of *Neisseria meningitidis* serotype antigens by immunodiffusion and SDS-polyacrylamide gel electrophoresis-immunoperoxidase techniques and the distribution of serotypes among cases and carriers. J. Gen. Microbiol. **116**:465–473.
17. **Poolman, J. T., C. T. P. Hopman, and H. C. Zanen.** 1982. Problems in the definition of meningococcal serotypes. FEMS Microbiol. Lett. **13**:339–348.
18. **Poolman, J. T., and H. C. Zanen.** 1980. Detection of antibody activity in human sera against meningococcal cell wall antigens using a gel-immuno-radio-assay (GIRA). FEMS Microbiol. Lett. **7**:293–296.
19. **Zollinger, W. D., and R. E. Mandrell.** 1977. Outer-membrane protein and lipopolysaccharide serotyping of *Neisseria meningitidis* by inhibition of a solid-phase radioimmunoassay. Infect. Immun. **18**:424–433.