Rapid serotyping of groups A, B, and C meningococci by rocket-line immunoelectrophoresis and co-agglutination

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SUMMARY Rocket-line immunoelectrophoresis (R-LIE) with antigen containing intermediate gel, and co-agglutination utilising protein A-containing staphylococci coated with specific antibodies, were adapted for serotyping the prototypes of group B meningococci. Both were found to have the same specificity as agar gel double diffusion (AGDD) but they were more sensitive and more rapid than AGDD. R-LIE required, like AGDD, the extraction of relatively large quantities of bacteria, while the co-agglutination method, performed as a slide agglutination test with results within a few minutes and no need of special equipment, required only a small amount of heated whole meningococcal cells. Meningococcal strains of serogroups B, C, and A from patients and carriers were serotyped and the results with all three methods were in agreement.

The serological grouping of *Neisseria meningitidis*, based on their specific capsular polysaccharide antigens, which are designated by tall letters (Branham, 1953; *Bergey's Manual*, 8th edition, 1974), has greatly aided epidemiological studies of meningococcal disease. A new approach to such studies was recently taken utilising subcapsular outer membrane antigens for division of groups B and C meningococci into distinct serotypes. Gold *et al.* (1971) utilised bactericidal tests with cross-absorbed antisera to heterologous group C meningococci which were separated into six serotypes designated by Roman numerals. In a series of studies, Frasch and Chapman (1972a, b; 1973) utilised bactericidal tests and precipitation in agar gel double diffusion to divide group B meningococci into distinct serotypes designated by Arabic numerals. Munford *et al.* (1975) recently showed that either system of Gold and Frasch could be used to type individual meningococcal strains of serogroups other than the prototype strain since the serotype antigen is independent of the serogroup one.

Munford *et al.* (1975) recommended agar gel double diffusion for routine qualitative tests as it was simpler to perform than the bactericidal tests, though less sensitive. Both, however, are laborious and time-consuming and may sometimes be difficult

to interpret.

In the present paper we describe the adaptation of rocket-line immunoelectrophoresis (R-LIE) and co-agglutination for serotyping meningococci. Both had the same specificity as agar gel double diffusion, and they were more rapid and more sensitive. Serotyping of groups B, C, and A meningococcal strains from patients and carriers gave with all three methods results that were in agreement.

Material and methods

MENINGOCOCCAL STRAINS AND CULTURE

Group B prototype strains representing serotypes 1 to 12 and type 15 (identification numbers, see Table 1) were kindly provided by Dr Carl E. Frasch, Bureau of Biologics, Food and Drug Administration, Bethesda, Md, USA. Twenty nonepidemic meningococcal strains were taken from the stock of our laboratory; 11 of them belonged to group B, seven to group C, and two to group A. They were from 13 patients with meningococcal disease and from seven carriers. The source of isolation and sensitivity to sulphonamide are given in Table 4.

The strains were cultured on BBL GC medium base enriched with Isovitalex, horse serum, and Bacto haemoglobin or on the gonococcal colony typing medium described by Kellogg *et al.* (1963). The strains were preserved as previously described (Danielsson and Sandström, 1978).

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Table 1 Results of agar gel double diffusion (AGDD) tests with saline extracts of meningococcal group B serotype prototype strains and their homologous and heterologous antisera

Rabbit antiserum against serotype prototype strain	Serotype specific precipitin bands in AGDD tests with saline extracts of meningococcal group B serotype prototype strains											
	1	2	2,7	2,10	4	5	6	8(1)	9	11	12	15
M1080/1	+											
B16B6/2		+	+	+								
M986/2,7		+	+	+								
M1011/2,10		+	+	+								
M981/4					+							
M992/5						+						
M990/6							+					
M978/8(1)	+							+				+
M982/9									+			
M136/11										+		
S-3032/12											+	
H-355/15												+

*Serotype specific precipitin bands showed reactions of partial identity.

ANTISERA AND ABSORPTION

Young rabbits were immunised with whole meningococcal cells by repeated intravenous injections over a period of five to six weeks. Two modes of antiserum production were used: one followed the procedure for antigenococcal antiserum described by Danielsson and Sandström (1978), the other the procedure described by Frasch and Chapman (1972a). The rabbits were bled 7-10 days after the last injection. Sera were preserved in the usual way in aliquots of 2 ml in the freezer (-20°C) or in the refrigerator at $+4^{\circ}\text{C}$ after addition of 1/10 000 merthiolate.

Sera to be used for co-agglutination were absorbed with appropriate group B prototype strains (Table 3). One volume of antiserum was mixed with one volume of meningococci (heated at 100°C for 30 minutes, washed once, and sedimented by centrifugation at 10 000 *g* for 20 minutes). The suspension was incubated at 37°C for 3-4 hours and in the refrigerator overnight. Absorbed sera were recovered by centrifugation, 10 000 *g* for 20 minutes, and stored in the freezer before use.

SALINE EXTRACTS OF SEROTYPE ANTIGEN

Saline extracts of the meningococcal strains were prepared as described by Frasch and Chapman (1972b).

AGAR GEL DOUBLE DIFFUSION

The Ouchterlony (1968) method was used. A 5-ml amount of melted 1% Special Noble Agar in 0.15 M saline was poured into small petri dishes (diameter 50 mm). Templates with a central well and six peripheral ones were used to punch holes (4 mm in diameter) in the agar 6 mm apart (centre to centre). In each petri dish four circular sets were cut. Undiluted antiserum was placed in the central well and undiluted (in a few experiments diluted 1:2, 1:4, and

1:8) or concentrated saline extracts in the peripheral wells. The reactions were read after one and two days.

ROCKET-LINE IMMUNOELECTROPHORESIS

R-LIE with antigen containing intermediate agar gel was carried out as described by Axelsen *et al.* (1973) using the micro modification with 5×5 cm glass plates. Miles agarose (Lot SC 3) was used throughout this study and was melted in barbital buffer (LKB, pH 8.6, ionic strength 0.025). Antigen wells, antigen containing intermediate gel, and reference antimeningococcal antibody gel were arranged as shown in Fig. 1, and the areas covered by gel measured approximately 7.5, 5, and 12.5 cm^2 , respectively, thickness 1.5 mm. The antigen wells, seven per plate, were each filled with 10 μl of undiluted or diluted saline extracts. The intermediate gel contained 20 μl of undiluted saline extracts per cm^2 , and the reference antibody gel 8 $\mu\text{l}/\text{cm}^2$ of undiluted antimeningococcal antiserum. An electrophoresis apparatus cooled with water at 15°C was used. The barbital buffer (LKB, pH 8.6, ionic strength 0.1) in the electrophoresis vessels was recirculated to minimise changes of the pH of the buffer. The R-LIE was run for 6 hours with a potential gradient of 6 V cm^{-1} or overnight with a gradient of 2 V cm^{-1} . After electrophoresis the precipitin reactions were read, the plates were then pressed under moist filter paper and soft blotting paper, washed, dried in the fan of a hairdrier, and finally stained with amido black as described by Axelsen *et al.* (1973).

CO-AGGLUTINATION PROCEDURES

The production and stabilisation of protein A-containing staphylococci to make co-agglutination reagents followed the procedures described by

Fig 1. Co-agglutination patterns obtained with reagent staphylococci coated with cross-absorbed serotype specific antibodies against *Neisseria meningitidis* group B prototype serotype strains. Heated whole meningococcal cells of serotype 1 in the wells of the horizontal row A, serotype 2 in row B, and serotype 4 in row C were mixed with reagent staphylococci coated with anti-type 1 in the wells of the vertical row 1, anti-type 2 in row 2, anti-type 4 in row 3, and anti-type 5 in row 4. Clearly visible ++ and +++ agglutination reactions are seen in wells A1, B2, and C3.

Kronvall (1973). For coating the staphylococci with antibodies, 1 ml of the 10% suspension was added to 0.1 ml of undiluted unabsorbed or absorbed rabbit antimeningococcal antiserum. After the suspension had been mixed, the staphylococci were washed twice and suspended to 1% in phosphate buffered saline (PBS, pH 7.2) with 0.1% NaN₃. In this way 10 ml reagents were obtained from 0.1 ml serum. Control reagent consisted of uncoated staphylococci. The suspensions were stable for at least four months when stored at +4°C.

Co-agglutination tests were performed with meningococcal organisms grown on the Kellogg medium (Kellogg *et al.*, 1963) for 20-24 hours, suspended in PBS, boiled for 20-30 minutes, washed once, and diluted to a 1% suspension. One drop of this suspension was mixed on a glass slide with one drop of reagent staphylococci. The slide was then tilted approximately once a second for 2 minutes and the reaction was read in oblique light. In some experiments saline extracts were used as antigen. Negative reactions were graded - or ±, weak reactions +, moderately strong ++, and strong or very strong +++. The ++ and +++ reactions usually started to appear within one minute and

gradually became stronger within the next minute. The weak reaction graded + usually took 2 minutes to appear.

Results

AGAR GEL DOUBLE DIFFUSION

Saline extracts of the prototype group B strains formed the characteristic serotype specific precipitin bands near the antigen well with their homologous antisera as described by Frascch and Chapman (1972b). The overall results, summarised in Table 1, deserve the following comments:

STA-2,7 and STA-2,10 gave reactions of partial identity with STA-2 in tests with anti-2. Partial identity was also demonstrated between STA-2/STA-2,7 and STA-2,10 in tests with anti 2,10. However, reactions of identity were obtained between STA-2/STA-2,10 and STA-2,7 in tests with anti-2,7. STA-1 and STA-15 gave reactions of identity with STA-8(1) in tests with anti-8(1), but the reverse was not obtained in tests with anti-1 and anti-15. The serotype specific precipitin band of the type 12 prototype strain was not formed until the saline extract was concentrated twice.

The saline extracts formed with most of the antisera a few additional precipitin bands that were unrelated to the serotype specific lines and always situated between these and the antibody wells. Antisera produced by the two different modes described in Material and methods were compared in the agar gel double diffusion tests but there were no obvious differences in the results with regard to the serotype specific and the additional precipitin bands formed.

ROCKET-LINE IMMUNOELECTROPHORESIS

R-LIE was performed as described in Material and methods with homologous and heterologous antigen-antibody combinations. The heterologous meningococcal saline extracts included as antigens in the intermediate agar gel corresponded to the serotype strains used for absorption in the co-agglutination tests (see below). Under these test conditions the homologous antigen-antibody combination forms a serotype specific rocket line (see Axelsen *et al.*, 1973), as illustrated in Fig. 2, which corresponds to the serotype specific precipitin band in agar gel double diffusion. Antigenically unrelated heterologous

strains will not form such rocket lines while those that are related will. The overall results are summarised in Table 2. It will be seen that the serotype specific rocket lines formed by the individual and homologous antigen-antibody combinations corresponded to the results in agar gel double diffusion tests. The close antigenic relationships demonstrated in agar gel double diffusion of the type 1 complex and between the extracts of serotypes 1,8, and 15 in tests with anti-8(1) were confirmed. The extracts of STA-5 and STA-8(1) also formed rocket lines with anti-1, which were differentiated from the rocket line formed by STA-1 by including extracts of types 4 and 8 in the intermediate agar gel.

The R-LIE tests could be read after 6 hours as compared to 24-48 hours for agar gel double diffusion. They were also more sensitive, that is, the extracts in R-LIE tests could be diluted four to eight times more than the same extracts used in agar gel double diffusion.

CO-AGGLUTINATION

Reagent staphylococci coated with unabsorbed anti-meningococcal antibodies gave strong reactions with their homologous strains (whole meningococcal cells boiled for 20-30 minutes) but also with most of the heterologous ones. Tests with K1 containing *Escherichia coli* were negative, and the reactions were therefore not due to the group B polysaccharide which is immunochemically identical with the K1 antigen (Glode *et al.*, 1977). However, reagent staphylococci coated with antibodies absorbed with organisms of the heterologous group B serotype strains reacted only with the homologous serotype prototype strains. Clearcut moderate (++) to strong (+++) agglutinations appeared within 1-2 minutes, and 12 tests on a test plate with circular rings were completed within less than 5 minutes. It will be seen from the summarised results in Table 3 that the cross reactions between serotypes 1, 5, and 8 in R-LIE tests with anti-1, and between types 1, 5, and 15 in both agar gel double diffusion and R-LIE were confirmed. However, absorption of anti-1 and anti-8 with meningococcal organisms of two serotypes made them specific for their homologous serotypes. Moreover, serotypes 2,7 and 2,10 were distinguished from others of the type 2 complex by absorption of their corresponding antisera with 2,10 and 2,7 respectively. The serotype specific reagent staphylococci were also tested with the saline extracts used in agar gel double diffusion and R-LIE. Strong agglutinations were obtained, and the results agreed completely with those of the whole cells. Moreover, the extracts could be diluted two to four times more than for R-LIE. Control tests with uncoated reagent staphylococci were always negative.

Fig. 2 Rocket-line immunoelectrophoresis with antigen containing intermediate agar gel. The rectangular area A contained agarose with anti-type 6 antiserum, and area B agarose with saline extract of serotype 9. Before the electrophoretic run ↑ basins 1-7 were filled with saline extracts: (1) serotype 1, (2) serotype 2, (3) serotype 9, (4) serotype 6, (5) serotype 4, (6) serotype 5, and (7) serotype 8. Serotype antigen 6 in basin 4 has formed with its homologous antiserum a serotype specific rocket line, the 'feet' of which go down into the antigen containing intermediate gel. The fused horizontal lines with peaks represent common antigens.

Table 2 Results of rocket-line immunoelectrophoresis with homologous and heterologous antigen-antibody combinations of meningococcal group B serotype prototype strains and with antigen containing intermediate gel

Rabbit antiserum against serotype prototype strain	Serotype antigen in intermediate agar gel	Serotype specific rocket lines in R-LIE tests with saline extracts of meningococcal group B serotype prototype strains											
		1	2	2,7	2,10	4	5	6	8(1)	9	11	12	15
M1080/1	4	+					+		+				
	4 and 8	+											
B16B6/2	4		+	+	+								
M986/2,7	4		+	+	+								
M1011/2,10	8		+	+	+								
M981/4	1					+							
M992/5	9						+						
M990/6	9							+					
M978/8(1)	5	+							+				+
M982/9	8									+			
M136/11	1										+		
S-3032/12	2											+	
H-355/15	8												+

Table 3 Results of co-agglutination of meningococcal group B serotype prototype strains with reagent staphylococci coated with serotype specific antibodies

Serotype prototype antibodies for coating reagent staphylococci	Serotype prototype strain used for absorption	Co-agglutination results with whole cells or saline extracts of meningococcal group B serotype prototype strains											
		1	2	2,7	2,10	4	5	6	8(1)	9	11	12	15
M1C80/1	4	+++					+++		+++				
M1080/1	4 and 8	+++											
B16B6/2	4		+++	+++	+++								
M986/2,7	4		+++	+++	+++								
M986/2,7	2,10			++									
M1011/2,10	8		+++	+++	+++								
M1011/2,10	2,7				++								
M984/4	1					+++							
M992/5	9						+++						
M990/6	9							+++					
M978/8(1)	5	++							+++				+++
M978/8(1)	5 and 15								++				
M982/9	8									+++			
M136/11	1										++		
S-3032/12	2											+++	
H-355/15	8												+++

++, +++ = moderate, strong co-agglutination, respectively. See Material and methods.

COMPARISON BETWEEN AGAR GEL DOUBLE DIFFUSION, R-LIE, AND CO-AGGLUTINATION FOR SEROTYPING MENINGOCOCCI ISOLATED FROM PATIENTS AND HEALTHY CARRIERS

The results of serotyping 20 group B, C, or A meningococcal strains by agar gel double diffusion, R-LIE, and co-agglutination are summarised in Table 4. They were all from sporadic cases with meningococcal disease or from healthy carriers. It will be seen from the Table that there was good agreement between all three methods. All group B strains were serotyped by the co-agglutination method, while three strains were not typed by agar gel double diffusion and one was not typed by R-LIE. Four group B strains reacted with co-agglutination reagents for two serotypes; this was

confirmed for three of them with R-LIE but not with agar gel double diffusion. Four out of seven group C strains were typable—three type 15 and one type 12,15. Only two group A strains were available, both of serotype 9. A family carrier strain (Cr-11Sv) of group B was of the same serotype as the index case (Pt-11). Among the sulphadiazine-resistant strains type 15 of serogroups B and C dominated.

Discussion

In the present work R-LIE was found to have the same specificity as agar gel double diffusion for serotyping prototype strains of group B meningococci. The sensitivity was increased four to eight times by R-LIE, and results were obtained one or two days

Table 4 Results of serotyping 20 meningococcal strains of serogroups A, B, and C by agar gel double diffusion (AGDD), rocket-line immunoelectrophoresis (R-LIE), and co-agglutination (COA)

Patient/Carrier (P-t) (C-r)	Meningococcal serogroup	Isolated from	Sulphonamide sensitivity	Serotypes demonstrated by:		
				AGDD	R-LIE	COA
P-t 105330	B	CSF	R	15	12,15	12,15
P-t 11-921	B	CSF	R	nt	12,15	12,15
P-t NF-51	B	Throat	S	2:2,7 2,10	2:2,7 2,10	2 only
P-t 3402	B	CSF	nd	2:2,7 2,10	2:2,7 2,10	2 only
P-t 5-793	B	Blood	R	15	15	15
P-t 1/11	B	Throat	S	nt	15	15
C-r SV/11	B	Throat	S	nt	nt	15
C-r 445	B	Throat	S	6	6	6
C-r 447	B	Throat	S	6	6	6,15
C-r 941	B	Throat	S	9	9	9,12
C-r 282	B	Throat	nd	9	9	9
P-t 5-226	C	Blood	R	15	12,15	12,15
P-t 5-338	C	Blood	S	15	15	15
P-t 11-877	C	CSF	S	15	15	15
P-t 11-114	C	CSF	S	15	15	15
P-t 11-467	C	CSF	R	nt	nt	nt
P-t 11-781	C	CSF	S	nt	nt	nt
P-t 5-245	C	Blood	S	nt	nt	nt
P-t 1-792	A	CSF	R	9	9	9
P-t 1-224	A	CSF	R	9	9	9

CSF = cerebrospinal fluid; nd = not done; nt = not typable; R = sulphonamide resistant; S = sulphonamide sensitive.

earlier than by agar gel double diffusion. However, we also found that serotyping could be performed as a simple and rapid slide agglutination test by using protein A-containing staphylococci coated with cross-absorbed serotype specific antibodies. This technique was found to be even more sensitive than R-LIE, and 12 tests could be done and read on a glass plate within less than 5 minutes. It required a minimum of bacteria as compared to agar gel double diffusion and R-LIE and could be done with whole cells as well as with saline extracts prepared according to Frasch and Chapman (1973). All three methods showed good agreement in tests of meningococcal strains from sporadic cases and carriers. In some strains two serotypes were demonstrated by R-LIE and co-agglutination tests, which could be due to the fact that these tests are more sensitive than agar gel double diffusion.

Jones and Tobin (1976) recently described the use of a tube agglutination test which they found to be satisfactory for serotyping group B meningococci. However, they reported that some strains were auto-agglutinable and could not be typed. The co-agglutination method has an advantage in this respect since it is not influenced by autoagglutinating bacteria (Christensen *et al.*, 1973; Danielsson and Kronvall, 1974; Olcén *et al.*, 1975). We also found that the co-agglutination method is sensitive enough to discriminate strains of the type 2 complex (types 2; 2,7; 2,10) which was previously done only by a

bactericidal inhibition technique (Frasch and Chapman, 1973).

Antisera to group B strains were successfully used by Munford *et al.* (1975) to serotype by agar gel double diffusion group C meningococci from sporadic cases and epidemic outbreaks. In this way they succeeded in typing 93 out of 107 strains. Correspondingly, we were also able to serotype group C strains with antisera against group B prototype strains and those typable belonged to type 15 or 12,15 like most of the carriers and sporadic cases with group B meningococci. This serotype has also been found among sporadic cases and in minor outbreaks in Norway (Holten, personal communication). It was of interest to note that the group B serotype 15 prototype strain gave reactions of identity with type 8 extracts in agar gel double diffusion tests, and serotype specific rocket lines in R-LIE, with anti-type 8(1) but not with the serotype 15 strains isolated from patients and carriers. Further studies have shown that the prototype 15 strain forms two serotype precipitin bands close to each other, one of which gives reactions of identity with the extracts of prototype 8(1).

There were only two group A strains available and both of them reacted with serotype 9 antibodies. This is of interest since homogeneity of the protein serotype antigens of group A meningococci was recently reported (Sippel and Quan, 1977). These authors found that they were distinct from group B

type 2 and group C type 2 but they did not study them against other group B serotype antibodies. Group A strains isolated from sporadic cases and in epidemic outbreaks therefore deserve further serotyping studies. The R-LIE and crossed-line immunoelectrophoresis have, in preliminary studies conducted by us, been found to be excellent tools for the further immunochemical characterisation of the serotype specific antigens of groups B and C as well as of group A meningococci (Danielsson and Olcén, in preparation).

The serotyping of groups B and C meningococci, the epidemiological significance of which has been well documented in several studies from the USA and the UK (Gold *et al.*, 1971; Frasch and Chapman, 1973; Farries *et al.*, 1975; Munford *et al.*, 1975; Jones and Tobin, 1976; Griffiss *et al.*, 1977), has given us new insights into the understanding of meningococcal disease. However, these studies have usually been carried out in retrospect, mostly because the agar gel double diffusion and bactericidal tests are not well suited in a running routine. In this respect the co-agglutination method offers an opportunity for a more continuous follow-up of the serotypes of meningococcal strains isolated from patients and from carriers with or without a connection with cases of the disease. The co-agglutination tests are also economical since 0.1 ml specific antiserum is enough to make up 10 ml reagent staphylococci, which will be sufficient for at least 150 to 200 tests.

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