

Method for Phage Typing Group A Type 49 Streptococci

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A method of phage subtyping group A type 49 streptococci is described. The method is similar to that used for phage typing staphylococci, except that lysates obtained by induction with mitomycin C rather than propagated stock phages were used. Five type 49 strains were used as phage donors. Seventy-two strains of type 49 streptococci isolated from 10 worldwide sources were examined by this method. Among these strains, five distinct subtypes (I through V) could be distinguished on the basis of their lytic patterns. Only a few of the type 49 strains could not be classified into one of these phage subtypes (6% using 100× routine test dilution). Strains from a single source were generally homogeneous with respect to their phage subtype. The method proved useful in discriminating between type 49 strains isolated from different geographical sources and from the same place in different years. Studies in progress suggest that it may be useful for subtyping other strains of special epidemiological interest, such as strains of other serological types associated with nephritis.

Most of the recent work on bacteriophages of group A streptococci has centered on their possible biological influences (18, 30) rather than on developing classification systems. This is understandable since (in contrast to staphylococci) reasonably good serological classification systems are available for group A streptococci. However, there are significant inadequacies in the serological typing systems of these organisms (22). Many strains are difficult to type, especially by the M system, resulting in attempts to classify group A streptococci by other type-specific substances such as the serum opacity factor (25, 29) or by the production of certain enzymes (23).

Early efforts to study and classify streptococci in relationship to bacteriophages utilized virulent phages and their associated lysins (see review by Maxted [20]). Lancefield (14) established the specificity of a virulent phage for group C streptococci. Evans and Verder (7) studied 257 strains of streptococci and were able to distinguish those of groups A, C, and E from those of groups B, D, F, G, H, and K based on the sensitivity of streptococci of the former groups to "nascent lysis" resulting from the presence of a phage-associated lysin (13, 19). Since over 97% of group A strains were resistant to a filtered preparation of this phage (containing no lysin), they could be differentiated from 90% of group C strains, which were sensitive to the filtered phage preparation.

Subsequent studies have shown that streptococci of groups A and C are relatively (12, 20), but not absolutely (2, 26), group specific with

respect to their susceptibility to virulent phages.

Efforts to further subdivide streptococci into types on the basis of reactions to virulent or temperate phages or to phage-associated lysins have met with variable success. Attempts by Evans (5) were made to divide 18 of Griffith's serological types into three clusters based on sensitivity or resistance to phage lysis and to nascent lysis by phage C/594. Some lytic patterns were evident, but the system was seriously handicapped by the use of only one phage. McKenna (17) reported his attempts to group and type 74 strains of streptococci using three of his own and four of Evans' (6) filtered virulent phages. Forty-three of the 60 group A strains could be identified as types 19, 17, or 3; 13 strains were not classifiable.

Kjems (10) tested four propagated temperate phages for lysis on 188 strains of group A streptococci representing 18 T-types. Lysis by either two or three phages was observed with strains from 9 of the 18 types in the study. Four types representing 34% of the strains were not lysed by any of the phages. Individual types could not be distinguished by the phage patterns.

In an attempt to link certain strains of streptococci to specific streptococcal diseases, Leonova et al. (15) applied 14 temperate bacteriophages, induced by ultraviolet light at routine test dilution (RTD) and distributed in five groups, to 327 group A streptococci, of which 170 or 52% were sensitive. Among 69 phage-sensitive strains from a total of 124 cultures from patients with nephritis and their families,

63 strains were lysed exclusively by one phage. All but four of the sensitive strains were type 12. Streptococcal strains isolated from cases of scarlet fever and tonsillitis were lysed by phages from four of the five phage groups. Strains from "rheumatism" were lysed by phages from all five phage groups.

In earlier work from this laboratory (28), temperate phages spontaneously released from several M types of group A streptococci were propagated and applied to a series of artificially lysogenized indicator lawns. Although successful in differentiating type 12 from type 49 strains and in distinguishing several type 49 phage patterns, this approach required the development of a set of discriminating indicator strains and its success depended upon obtaining and propagating phages from the strains to be tested, which was sometimes difficult or impossible to achieve.

In the present report, two approaches to identifying and subclassifying group A streptococci of certain M types have been explored and compared. First, the system of prophage typing was examined further by applying induced lysates of test strains directly to a set of natural indicator strains. Second, a set of induced lysates from selected strains was used to distinguish specific lytic patterns on lawns of test strains. The latter method proved useful for subtyping streptococci of type 49 and in preliminary studies of type 12 streptococci.

MATERIALS AND METHODS

Strains. The M type 12 and type 49 strains of group A streptococci used in these studies have been previously described (21, 28). The strains represented a variety of epidemiological incidents from a number of geographical sources. Type 49 strains, which gave weak or no reactions with type-specific M antisera, were identified by specific T agglutination and serum opacity inhibition reactions (21, 25, 29). Type 49 strains were obtained from Minneapolis, Red Lake, and Ponemah (Minn.), New York, Chile, The Netherlands, Czechoslovakia, Alabama, Trinidad, and Britain (see references in Table 3).

Media. The liquid medium used in these experiments was no. 1 broth (26). The agar plates, designated as R6, were a modification of the broth using 3% proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.), 0.6% NaCl, 0.02% CaCl₂, 0.07% Na₂HPO₄, 5% horse serum, 0.1% glucose, 1% agar (Difco), and hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 68 µg/ml. The medium was prepared and autoclaved without the CaCl₂, glucose, horse serum, and hyaluronidase. Concentrated solutions of CaCl₂ and glucose were sterilized by autoclaving; a concentrated solution of hyaluronidase was sterilized by filtration. These were added along with sterile horse serum to the autoclaved medium, cooled to 45°C, to obtain the final concentrations as indicated.

Preparation of test lawns. All strains or group A streptococci were stored in blood broth at -20°C. One drop of an overnight 34°C blood broth culture was added to 5 ml of no. 1 broth and incubated overnight at 30°C. The next day the culture was mixed vigorously, diluted 1:20 in no. 1 broth, and flooded on R6 agar plates, and the excess fluid was removed. The plates were partially dried on the bench. After further drying for 15 to 20 min at 34°C (28), dilutions of phage were applied. The plates were incubated at 34°C overnight and examined the following morning for plaque titer.

Induction of phage from stock strains by mitomycin C. One drop of culture from a fresh blood broth was added to 5 ml of no. 1 broth and incubated overnight at 30°C. One-tenth milliliter was added to 2 ml of no. 1 broth containing 0.1% glucose. The culture was incubated for 1 h in a 37°C water bath. Mitomycin C (Sigma) was added to a final concentration of 0.1 µg/ml, and incubation was continued for an additional 2 h at 37°C. The cells were centrifuged, and the supernatant was filtered through a 0.45-µm membrane filter (Millipore Corp., Bedford, Mass.) (26, 28).

Multiple application of phage lysates to test lawns. The freshly prepared mitomycin-induced phage filtrates were applied to lawns of all the test strains in log₁₀ dilutions (undiluted to 10⁻⁴) with a Lidwell phage-typing machine (Biddulph & Co., Manchester, England) (16).

TD. An RTD was established for each of the phage lysate preparations. The stock strains from which the phages were induced for subtyping were screened, and those that were sensitive were chosen for determining the RTD. For each phage preparation, the highest dilution that gave near confluent lysis was used as the RTD (1).

Lytic spectra. Lytic spectra were determined, and reactions were graded according to a system similar to that described for coagulase-positive staphylococci (1). The reactions of each phage on the test strain were compared to its RTD, and the log difference in titer was expressed as a phage reaction number (see footnote a, Table 1). A phage reaction with a titration of 50 or more plaques in the same dilution as RTD was assigned a phage reaction number of 5. A reaction of 50 or more plaques for each log₁₀ dilution more concentrated than RTD was given one number lower (28). No lysis with undiluted lysates was designated as NL. Inhibition reactions manifested as weak lysis with no plaques at any dilutions were designated 0. Reactions on strains or groups of strains that appeared as inhibition in some experiments and plaques in others were recorded with multiple designations, e.g., 0/2 (i.e., inhibition in some experiments and 50 or more plaques at a dilution 10³ more concentrated than RTD in other experiments).

RESULTS

Subtyping by prophage-typing method. The prophage-typing method was performed by the application of induced lysates from test strains to a set of five specific indicator strains. A series of lytic patterns was obtained which per-

mitted classification of the prophage, and thus the donor type 49 strain, into subtypes. However, it became apparent that prophage typing had serious limitations when 29 of the 72 type 49 strains (strains from Chile, Britain, Trinidad, and some from Alabama and Czechoslovakia) yielded few or no plaque-forming units when induced with mitomycin C.

Subtyping by the phage-typing method. For the phage-typing method a set of five induced phage lysates was applied to lawns of the 72 test strains, and only one strain at the undiluted phage concentration and six strains at RTD were unable to demonstrate a satisfactory lytic pattern. This approach greatly increased the chance of obtaining a lytic pattern as compared to the prophage-typing method.

Comparison of lytic patterns obtained with induced lysates applied in various dilutions. The nature of the lytic reactions was explored by examining dilutions of the lysates for the appearance of plaques. Four of the five phage lysates were titered out by simultaneous application of \log_{10} dilutions, from undiluted to 10^{-4} , to test lawns with the Lidwell phage-typing machine. (One of the five lysates in the original set was eliminated when it was found to lyse most of the strains in all of the subtypes at RTD.) An RTD was established for each lysate preparation, and the reactions on the test strains were expressed by reaction numbers, ranging from 0 to 5 (see Materials and Methods). Lytic reactions obtained on five representative type 49 strains are shown in Table 1. Reactions with individual strains were generally reproducible within 1 log or less. On the basis of the lytic patterns obtained on these representative strains, they were designated as subtypes I through V.

A comparison was made of the lytic patterns obtained on the 72 test type 49 strains with induced lysates applied in various dilutions

(Table 2). At RTD only 25 of 72 strains (35%) gave lytic patterns that fell into one of the five subtypes. Forty-seven strains did not react with or gave miscellaneous patterns with the phage lysate. Therefore, the RTD did not appear to be a useful dilution for discriminating among these strains.

On the other hand, at $100\times$ RTD, 68 (94%) of the test strains exhibited patterns represented by the five subtypes. Three strains showed no phage lysis and one strain gave a miscellaneous reaction.

The undiluted lysate placed 62 of the 72 strains, or 86%, into the five subtypes. Seven strains (two from Alabama and five from Czechoslovakia) demonstrated lysis or inhibition from all five phage lysates. One strain gave no phage pattern, and two strains classified as miscellaneous gave variations of a subtype. Although superficially there appears to be good correlation between lytic patterns at $100\times$ RTD and undiluted lysates (Table 2), 16 (22%) of the strains showed different lytic patterns

TABLE 2. Distribution of lytic patterns exhibited by 72 type 49 strains when tested with three concentrations of phage lysates

Lytic patterns ^a	Sub-type	No. of strains		
		RTD ^b	$100\times$ RTD	Undiluted ^c
2/4	I	2	21	21
1/3/4	II	4	15	15
1/2/4	III	6	16	17
1	IV	8	13	4
1/4	V	5	3	5
1/2/3/4		0	0	7
None or miscellaneous		47	4	3

^a See Table 1 for strain source of lysates.

^b Phage lysate concentration.

^c 10^3 to $10^4\times$ RTD.

TABLE 1. Lytic reactions^a of induced phage lysates on representative type 49 strains

Representative test strain	Reaction from lysate:				Lytic pattern at $100\times$ RTD ^b	Subtype
	1 (from strain GT8760)	2 (from strain GT7907)	3 (from strain GT9278)	4 (from strain GT7903)		
GT8760	NL	4	NL	3	2/4	I
GT7907	5	NL	5	5	1/3/4	II
GT9278	4	5	NL	4	1/2/4	III
GT7903	4	NL	NL	NL	1	IV
GT6256	5	0/2	0/2	5	1/4	V

^a Five, 50 or more plaques in the same dilution as RTD; 4, 50 or more plaques in dilution 10 times more concentrated than that of RTD; 3, 50 or more plaques in dilution 10^2 times more concentrated than that of RTD; 2, 50 or more plaques in dilution 10^3 times more concentrated than that of RTD; 1, 50 or more plaques in dilution 10^4 times more concentrated than that of RTD. NL, No lysis with undiluted lysates. 0, Inhibition; 0/2, inhibition/phage lysis, i.e., variable results for different experiments.

^b Numbers indicate lysates giving 50 or more plaques at $100\times$ RTD.

when examined with these two dilutions. However, 15 of these strains were from two sources (Alabama and Czechoslovakia), whereas the strains from six other sources were remarkably unchanged.

Association of phage subtypes with source and nature of epidemiological incident. The 72 type 49 strains represented a variety of incidents from different geographical locations, some associated with nephritis and others not associated with nephritis (Table 3).

Type 49 strains from the Red Lake outbreak of 1966 were classified as lytic pattern 2/4 (subtype D); those from patients with and without nephritis could not be differentiated by their lytic patterns. A number of type 49 strains were isolated from Ponemah, a village about 30 miles distant from the town of Red Lake, during or in the year after the 1966 outbreak, but less evidence of an increased incidence of hematuria or nephritis was detected in this population (B. F. Anthony, E. L. Kaplan, L. W. Wannamaker, and S. S. Chapman, *Am. J. Epidemiol.*, in press). The type 49 Ponemah strains had the same 2/4 lytic pattern as those isolated from individuals in the town of Red Lake.

It was of interest that the lytic pattern obtained with type 49 strains from the Red Lake nephritis outbreak of 1966 (pattern 2/4) differed from that obtained with type 49 strains obtained in the Red Lake nephritis epidemic of 1953 and the related Minneapolis family outbreak (lytic pattern 1; subtype IV). Both of these phage subtypes (I and IV) were repre-

sented in strains collected in Alabama in association with sporadic cases of acute nephritis during 1964 and 1965, the interim period between the two outbreaks at Red Lake.

Lytic pattern 1/3/4 (subtype II) included strains from The Netherlands and Chile where nephritis was occurring. Strains from Great Britain, for which there was no reported connection with glomerulonephritis, had an identical lytic pattern. Thus, no separation could be made here between strains from areas with and without reported nephritis.

Type 49 strains from Trinidad all gave a distinctive pattern (lytic pattern 1/2/4; subtype III). Similar strains were found in the Alabama group and in one strain from the Rockefeller collection.

All of the strains from each incident or location were surprisingly homogeneous with respect to subtype except for strains from Alabama and Czechoslovakia. The Alabama strains were from a heterogeneous population of patients with sporadic nephritis which was large enough to maintain several subtypes. It is uncertain whether the Czechoslovakia strains used in these studies are from one small epidemic or whether some of them were collected from sporadic infections throughout the country.

DISCUSSION

With continued improvement of techniques for working with temperate bacteriophages of group A streptococci, it became apparent that

TABLE 3. Distribution of phage-subtyping patterns for 72 type 49 strains by source

Place or source	Year	Nephritis ^a	Reference	No. of strains of phage pattern (100× RTD) and subtype ^b :					
				2/4 I	1/3/4 II	1/2/4 III	1 IV	1/4 V	None or miscel- laneous
Red Lake Reservation									
Town of Red Lake	1966	+	9	7					
Town of Red Lake	1966	-	9	3					1
Village of Ponemah	1966-1967	±	- ^c	4					
Village unspecified	1953	+	11				1		
Minneapolis family	1953	+	27				3		
Great Britain	1966?	-	21		4				
The Netherlands	1960-1962	+	3		4				
Chile	Unknown	+	28		7				
Rockefeller University ^d	Unknown	Unknown	8, 21			1			
Trinidad	1965-1966	+	21			13			
Alabama	1964-1965	+	4	7		2	6		1
Czechoslovakia	1961-1962	+	24				3	3	2

^a Nephritis present (+) or absent (-) in patient or in population (see text).

^b At 100× RTD none of the strains exhibited the 1/2/3/4 lytic pattern (see Table 2).

^c B. F. Anthony, E. L. Kaplan, L. W. Wannamaker, and S. S. Chapman, *Am. J. Epidemiol.*, in press.

^d Strain C171/100/3 in Lancefield's collection, originally isolated by Ann Kuttner and designated 24 Berg. This was typed as M35, which is now considered to be identical with M type 49 (8, 21).

under appropriate conditions most type 12 and 49 strains will make excellent lawns. Moreover, many of the strains that produced few if any phage spontaneously in the culture supernatant gave a good yield, up to 10^9 plaque-forming units/ml, when induced with mitomycin C. It therefore seemed appropriate to explore the possibility of developing a more definitive system for further subdividing strains of streptococci of special interest, such as the nephritogenic types. Similarities or differences in strains from different incidents or from different families, populations, or individual patients, with and without renal complications, may provide further tags or clues about the epidemiology of acute nephritis. A phage-typing system developed as a modification of techniques used in earlier studies of temperate phages of nephritogenic strains (28) was found to be of limited general usefulness because of the difficulties in inducing phage production in a significant number of strains (40% of the 72 type 49 strains examined in this study). A more conventional phage-typing system, in which freshly induced lysates rather than stocks of propagated phages were used, proved to be convenient, reproducible, and successful in classifying a high percentage of strains (94% with $100\times$ RTD).

Simplicity is the unique part of this method in that only stock streptococcal strains need to be maintained instead of high-titer phage stocks. Results are easily obtained by using phage undiluted or at $100\times$ RTD. This method may allow even laboratories that are not highly specialized to phage type group A streptococci without the need for maintaining a complete stock of high-titer phages or a central reference laboratory for dispensing phages.

Group A temperate phages differ from staphylococcal phages in that phage filtrates rapidly lose titer in the cold room at 5°C even during a week of storage, whereas staphylococcal phages may be stored for weeks with little or no drop in titer. Storage of filtrates of temperate phages from group A streptococci at -20°C often gave variable results; the loss of titer was slowed down for some phages, whereas others showed almost complete loss of titer after 1 or more weeks of storage at this temperature. Attempts have not been made to study longevity of the phage at -70°C .

This method of phage subtyping is similar to staphylococcal phage typing, except that staphylococcal phage stocks are produced by infection, whereas the streptococcal phage preparations used here were freshly induced by mitomycin C from stock bacterial strains. Possible

advantages of the induction system, in addition to circumventing the problem of instability on storage, include the elimination of host-induced modification of the phage, which may occur when phages are propagated on stock strains, and the elimination of carry-over of phage from stock propagating strains that may be lysogenic. This carry-over phage may not be evident at RTD but might manifest itself at lower dilutions when applied to sensitive test lawns.

The use of undiluted lysates instead of $100\times$ RTD would obviously further simplify this phage-typing system. However, the reproducibility and comparability of the phage titers in induced lysates from all donor strains would need to be assured before undiluted lysates should be recommended for general use. Also, the use of undiluted lysates may increase the possibility of lysis from without by high multiplicity of phage, by cell wall-lysing enzymes, or by phage fragments. Surprisingly, in our experiments all of the type 49 strains, except some of the Alabama and Czechoslovakia strains, were completely free of inhibition or lysis at lower dilutions. The strains that did demonstrate lysis at low dilutions often produced plaquing to a variable degree in different experiments. This may be a situation where there is a lowered plaque titer due to a host restriction mechanism, heterologous host deoxyribonucleic acid, special nutritional requirements for the strains in question, or adsorption problems.

Although there was a shift of 16 strains (22%) from one subtype to another when comparing patterns at $100\times$ RTD and undiluted, the strains still fell into the same lytic patterns (subtypes I through V), except for the seven strains sensitive to all five phage lysates. Weak phage reactions that were passed over at $100\times$ RTD accounted for the differences in patterns obtained with undiluted lysates.

This method of using freshly induced lysates presents the possibility that the lysate may contain multiple phages if the donor stock strain has more than one inducible prophage (28). Multiple phages in a lysate may lower the number of phage types since the lysate would be acting as a phage pool rather than as a single phage, thus having the potentiality of lysing more strains. Perhaps in this respect the method used in the staphylococcal phage-typing system would be superior since single plaque isolates are propagated to high titer by infection, thus minimizing the possibility of lysates of heterogeneous composition.

By the use of this phage-subtyping system, the relationship of type 49 strains from different sources could be examined. Two outbreaks

of nephritis at Red Lake associated with type 49 streptococci and separated by an interval of 13 years proved to be of distinctly different phage subtypes, indicating an interesting biological change in this nephritogenic strain. Type 49 strains isolated from sporadic cases of nephritis in Alabama in the years immediately before the second Red Lake outbreak included representatives of both of these phage subtypes. The lytic pattern of some type 49 strains associated with nephritis in Czechoslovakia occurring during the interim between the two episodes at Red Lake resembled those found in strains recovered at the time of the first outbreak at Red Lake. Type 49 nephritogenic strains from Trinidad were of a distinctly different phage type from those isolated from either episode at Red Lake. Another lytic pattern was exhibited by all type 49 strains examined from Great Britain, The Netherlands, and Chile but was not found among strains isolated from any other source. Still another pattern was found only among strains isolated from Czechoslovakia. In general, the lytic patterns obtained on strains from one geographical source at one time were homogeneous. The type 49 strains from Alabama and Czechoslovakia were more heterogeneous, perhaps reflecting collection from sporadic cases or from unrelated mini-outbreaks. The presence or absence of nephritis in individual patients or in populations could not be related to differences in phage subtype.

Further studies are needed to determine whether this method of phage typing of type 49 strains can be extended to group A streptococci of other serological types. However, preliminary studies with type 12 streptococci indicate that this can be done with other strains of special epidemiological interest.

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