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Phage Typing Scheme for Group D Streptococci Isolated from Human Urogenital Tract

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Streptococci of Lancefield group D were isolated from 52.2% of pharyngeal, 55.5% of urethral, 56.6% of endocervical, and 75.4% of ano-rectal specimens. Seventeen phages isolated from sewage and urogenital specimens were selected for phage typing. Four of these lysed only the strains of *Streptococcus faecalis* and its variety *liquefaciens*. Another six phages lysed only the strains of *Streptococcus faecium* and its variety *durans*. With the help of seven bacteriophages, 346 of 384 (90.1%) strains of *S. faecalis* and its variety *liquefaciens* could be classified into 27 types. Similarly, with the help of 10 other bacteriophages, 97 of 117 (82.9%) strains of *S. faecium* and its variety *durans* could be grouped into 22 types. In 72 of 87 (82.7%) cases, similar phage types were obtained at different culture sites of the same individual.

The presence of temperate bacteriophages in enterococci was reported as early as 1922 by Beckerich and Hauduroy (4). The specificity of temperate or virulent phages was reported by Evans (12), Evans and Sockrider (13), Kjems (18) and Timperley et al. (24). Ciucă et al. (8) attempted a phage typing of 400 strains of group D streptococci with the help of four serologically distinct phages isolated from sewage. Hoch and Herman (17) isolated 14 virulent and 11 temperate phages to type 259 strains of enterococci untypable with Ciucă phages.

Though group D streptococci are generally considered of low pathogenicity, their role in subacute bacterial endocarditis, colitis, colicystitis, meningitis, osteomyelitis, abscesses, and urinary tract infections is known (10, 15, 25, 26). Because of their common habitat in human and animal intestinal tracts, they are also considered as one of the indicators of fecal pollution in water and food.

The present study was undertaken to establish a phage typing scheme for group D streptococci strains isolated from the urogenital tract, rectum and pharynx and to evaluate its application for ecological and taxonomical purposes.

MATERIALS AND METHODS

Bacterial strains. Five hundred and one strains of enterococci were isolated from 843 swabs taken from pharynx, urethra, cervix, or rectum of 361 cases seen at a hospital, a venereal disease clinic, local health units, or at physicians' private offices. Specimens received in Stuart transport medium (22) for routine investigation of *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, and *Candida albicans* (3) were used for the isolation of group D streptococci. Streptococci were isolated on enterococcus agar (Difco) and identified biochemically (9) and classified serologically as belonging to group D by the hot hydrochloric acid method of Lancefield (19). Streptococcus group D antisera lot no. 2743-50 (Difco) was used for precipitin test.

Bacteriophages. Bacteriophages were isolated according to the method of Adams (2). The specimen swab or the undiluted sewage was inoculated into brain heart infusion broth (Difco) and incubated at 37 C for 18 h. The culture was centrifuged and filtered through a 0.45-μm membrane filter (Millipore Corp.) and the filtrate was screened for the presence of bacteriophages by putting drops on preseeded lawns of indicator bacteria and observing the lysis. Phage purification was carried out by three or more consecutive single plaque propagations. Final propagation and preparation of high-titered lysates was done by the soft agar layer technique (23). Of a total of 51 phages, 5 isolated from urogenital specimens and 12 from raw sewage were selected for phage typing on the basis of plaque and particle morphology and host range activity. The phages in routine test dilution, the highest dilution which gave a confluent lysis, were used for phage typing. Heat inactivation studies of the phages were performed by exposing 1-ml aliquots of diluted phage suspensions to 60, 65, and 70 C for 30 min in a shaking water bath and by comparing the number of surviving plaque-forming units with that of the corresponding unheated phage suspension.

Phages were designated by the number of the sewage or the urogenital specimen with the denominator representing its propagating strain. All phages isolated from urogenital specimens were preceded by the letters VD. For example, phage

22/StJ1 and VD13/8413 were isolated, respectively, from sewage sample number 22 and urogenital specimen number VD13 and were propagated on strains StJ1 and 8413, respectively. For simplicity, the phages were given serial numbers in the text.

RESULTS

Frequency of isolation of group D streptococci. Of a total of 843 specimens from pharynx, urogenital, and ano-rectal sites of 361 cases, 501 (59.4%) were positive from group D streptococci (Table 1). The distribution of cases was: 65 from a juvenile delinquent custodial home, 63 from a general hospital, 41 from university students, and 192 from general practitioners throughout the province. A case was represented as positive if group D streptococci were isolated from any one or more of the culture sites.

Characteristics of typing phages. The presence of lysogeny among the strains was studied according to the technique of Fisk (14). Lysogeny could not be demonstrated by using the routine indicator strains. Induction by ultraviolet or mitomycin C was not studied. A total of 51 bacteriophages, one belonging to a new morphological type (1), was isolated from sewage and urogenital specimens. Table 2 represents certain characteristics of the 17 phages selected for phage typing. Plaque size varied from <50 μm to >250 μm in soft agar. Their titers ranged from 10^4 to 5×10^9 plaque-forming units per ml. All phages were inactivated at 70 C after 30 min of exposure (Table 3). Streptococcal group D phages were quite stable and high-titered stocks could be maintained for 2 years at 4 C without any appreciable loss in titer.

TABLE 1. *Frequency of isolation of group D streptococci from specimens of four different sites of 361 cases*

Sites	No. of specimens received	No. of specimens positive	Positive (%)
Pharynx	132	69	52.2
Urethra	270	150	55.5
Endocervix	270	153	56.6
Ano-rectum	171	129	75.4

Host range activity of bacteriophages. The host range activity appeared to be limited to strains of streptococcal group D. Ten strains each belonging to genera *Salmonella, Shigella, Staphylococcus, Pseudomonas, Klebsiella, Proteus, Enterobacter, Neisseria, Bacillus,* and *Streptococcus* (groups A, B, and C) did not show any lysis to enterococcal phages.

Phage typing. For phage typing purposes, bacteriophages were grouped into two sets. The first, used for phage typing of *S. faecalis* strains and its varieties, contained seven bacteriophages, namely 4, 5, 6, 17, 1, 14, and 15, of which 4, 5, 6, and 17 lysed only strains of *S. faecalis*, *S. faecalis* var. *liquefaciens* and *S. faecalis* var. *zymogenes*, whereas phages 1, 14 and 15 lysed, in addition, strains of *S. faecium* and its vari-

TABLE 2. *Origin, titer, and plaque size of typing phages*

Phage no.	Designation (phage/propagating strain)	Origin	Titer (PFU/ml)[a]	RTD[b]	Plaque size[c]
1	1/V12	Sewage, Ste-Rose	5.4×10^9	10^{-3}	S
2	6/46	Sewage, Ste-Rose	7×10^6	10^{-3}	ES
3	7/46	Sewage, Ste-Emilie	7×10^6	10^{-4}	L
4	13/3	Sewage, Ste-Rose	2×10^8	10^{-3}	M
5	14/4	Sewage, Ste-Rose	1.5×10^9	10^{-4}	S
6	28/11	Sewage, Ste-Rose	1×10^8	10^{-3}	M
7	29/904ED	Sewage, Laval	1×10^8	10^{-3}	ES
8	50/4301	Sewage, St-Janvier	1.5×10^8	10^{-2}	S
9	10/St-J3	Sewage, St-Janvier	4×10^4	10^{-1}	L
10	27/St-J3	Sewage, St-Janvier	2.6×10^9	10^{-5}	L
11	47/8736	Sewage, St-Janvier	2×10^7	10^{-1}	M
12	73/8073	Sewage, St-Janvier	1×10^8	10^{-2}	M
13	VD1/77	Vagina F25	1×10^5	10^{-2}	S
14	VD6/159	Cervix F31	1×10^6	10^{-3}	S
15	VD13/8413	Cervix F22	1×10^6	10^{-3}	L
16	VD22/1260	Cervix F42	9.5×10^4	10^{-2}	L
17	VD1884/3854	Urethra F25	5×10^5	19^{-3}	L

[a] PFU, Plaque-forming units.
[b] RTD, Routine test dilution.
[c] L, 250 μm and over; M, 100 to 250 μm; S, 50 to 100 μm; ES, <50 μm.

ety *durans*. Table 4 represents the lytic patterns of their propagating strains. The second set, composed of 10 bacteriophages, namely 2, 3, 7, 13, 8, 9, 10, 11, 12, and 16, was used for typing strains of *S. faecium* and its variety *durans*. Phages 8, 9, 10, 11, 12, and 16 were specific to *S. faecium* and its variety *durans*. Phages 2, 3, 7, and 13 lysed also strains of *S. faecalis* group. Table 5 represents the lytic patterns of their propagating strains.

As shown in Table 6, 346 of 384 (90.1%) of strains of *S. faecalis* and its variety *liquefaciens* could be grouped into 27 phage types. Phage types 5, 18, and 25 were the most common. Table 7 represents the lytic patterns of *S. faecium* strains and its variety *durans*. Ninety-seven of 117 (82.8%) strains could be grouped into 22 phage types. Phage types 4, 7, and 17 were the most common.

Agreement between the phage types. To establish a correlation between the phage types, streptococcal group D strains isolated

TABLE 3. *Heat inactivation of the typing phages*[a]

Phage		Inactivation (%) after 30 min of exposure at:					
		60 C		65 C		70 C	
No.	PFU/ml at 37 C	PFU/ml	Inactivation (%)	PFU/ml	Inactivation (%)	PFU/ml	Inactivation (%)
1	3×10^8	2×10^8	33.4	0	100.0	0	100.0
2	6×10^6	1×10^6	83.4	0	100.0	0	100.0
3	1×10^6	1×10^6	0.0	1×10^2	99.9	0	100.0
4	4×10^8	4×10^8	0.0	0	100.0	0	100.0
5	5×10^8	4×10^8	20.0	2×10^8	60.0	0	100.0
6	10×10^7	1×10^7	90.0	0	100.0	0	100.0
7	2×10^8	1×10^8	50.0	0	100.0	0	100.0
8	1×10^6	2×10^5	80.0	0	100.0	0	100.0
9	1.2×10^8	1×10^6	99.2	0	100.0	0	100.0
10	4×10^4	1×10^4	75.0	0	100.0	0	100.0
11	1×10^8	2×10^7	80.0	0	100.0	0	100.0
12	1×10^8	1×10^8	0.0	0	100.0	0	100.0
13	2×10^5	1.6×10^5	20.0	0	100.0	0	100.0
14	2×10^6	1×10^6	50.0	0	100.0	0	100.0
15	1×10^6	1×10^5	90.0	0	100.0	0	100.0
16	2×10^4	2×10^4	0.0	0	100.0	0	100.0
17	1.5×10^4	1×10^4	33.4	0	100.0	0	100.0

[a] PFU, Plaque-forming units.

TABLE 4. *Lytic spectra of Streptococcus faecalis bacteriophages on their propagating strains*

Propagating strain	Bacteriophage[a]						
	4	5	6	17	1	14	15
Streptococcus faecalis var. *liquefaciens* (3)	CL	CL	CL				CL
S. faecalis var. *zymogenes* (4)		CL	CL	CL	CL		
S. faecalis (11)	CL	CL	CL				
S. faecalis (3854)	CL	CL		CL			
S. faecalis (V12)					CL		
S. faecalis var. *liquefaciens* (159)						CL	CL
S. faecalis (8413)	CL	CL	CL			CL	CL

[a] CL, Confluent lysis.

TABLE 5. *Lytic spectra of Streptococcus faecium bacteriophages on their propagating strains*

Propagating strain	Bacteriophage[a]									
	2	3	7	13	8	9	10	11	12	16
Streptococcus faecium (46)	CL	CL			CL	CL			CL	CL
S. faecium (904ED)	CL	CL	CL		CL					
S. faecium var. *durans* (77)			CL	CL						
S. faecium (4501)	CL		SCL		CL	—	CL	CL	CL	
S. faecium (StJ3)		CL	CL		CL	CL	CL	CL	CL	CL
S. faecium (8736)					CL			CL		
S. faecium (8073)	CL	CL			CL	CL			CL	
S. faecium (1260)	CL	CL			CL	SCL	CL	CL	CL	CL

[a] Abbreviations: CL, Confluent lysis; SCL, semiconfluent lysis.

TABLE 6. *Phage typing of Streptococcus faecalis and its variety liquefaciens*[a]

Phage types	Phage types							No. of strains
	4	5	6	17	1	14	15	
1	CL	CL	CL	CL		CL		2
2	CL	CL	CL	+++	CL		CL	9
3	CL	CL	CL		CL	CL	CL	8
4	CL	CL	CL			CL	CL	1
5	CL	CL	CL				CL	71
6	CL	CL	CL					27
7	CL	CL		CL				7
8	CL	CL				+		29
9	CL	CL					CL	16
10	CL	CL			CL			2
11	CL	CL						3
12	++				CL			5
13	CL							1
14		CL	CL					3
15		CL		CL		CL		5
16		CL		—			CL	7
17			CL	—	CL		CL	8
18			CL	—			CL	40
19			CL	—	CL			9
20			CL	—				12
21				CL				1
22					CL	CL	CL	5
23						CL	CL	1
24						++		7
25					CL		CL	47
26							CL	14
27					CL			6
NT								38

[a] Abbreviations: NT, Nontypable, ++, 20 to 50 plaques; +++, more than 50 plaques; +, less than 20 plaques; CL, confluent lysis.

from different sites (pharynx, urethra, endocervix, and ano-rectum) of 87 females were examined. In 72 of 87 (82.7%) cases the same phage types were isolated from different sites of the same patient (Table 8). Of the 15 cases which did not show a complete agreement in phage

TABLE 7. *Phage typing of Streptococcus faecium and its variety durans*[a]

Phage types	Phage types										No. of strains
	2	3	7	13	8	9	10	11	12	16	
1	CL	CL	CL		CL						1
2	CL	CL			CL	CL	CL	CL	CL	CL	1
3	CL	CL			CL	CL			CL		1
4	CL	CL			CL	CL			CL	CL	10
5	CL	CL			CL	CL					1
6	CL	CL			CL		CL		CL		1
7	CL	CL			CL			CL	CL		10
8	CL	CL				CL			CL		5
9	CL	CL				CL					2
10	CL	CL							CL		4
11	CL		CL		CL		CL	CL	CL		5
12	CL			CL							4
13		CL	CL		CL	CL	CL	CL	CL	CL	4
14			CL	CL	CL				CL		3
15			CL	CL							1
16								CL	CL		5
17					CL		CL	CL	CL		14
18					CL			CL	CL		4
19					CL			CL			5
20					CL				CL		5
21					CL						5
22									CL		5
NT											21

[a] Abreviations: NT, Nontypable; CL, confluent lysis.

TABLE 8. *Correlation between the phage types of streptococcal group D strains isolated from different sites*

S. faecalis		Complete agreement on all sites explored[a]				*S. faecium*	
No. of cases	Phage types encountered	P	U	C	R	No. of cases	Phage types encountered
9	5, 6, 20, 26	+	+	+	+	1	11
4	5, 6, 26, 27	+	+	+	−	—	—
7	6, 11, 20, 26	−	+	+	+	—	—
8	2, 5, 10, 11, 18	+	+	−	+	3	19, 21
3	19, 20, 25	+	−	+	+	2	9, 12
5	6, 20, 27	−	−	+	+	1	20
19	6, 11, 13, 19, 20, 27	−	+	+	−	3	8, 20, 21
7	6, 11, 14, 19, 25	−	+	−	+	—	—

[a] Abbreviations: P, pharynx; U, urethra; C, endocervix; R, ano-rectum; +, sites from which streptococci of the same phage types were obtained; −, sites from which specimens were not taken.

types isolated from different sites, 11 showed a disagreement at one site, 3 at two sites, and 1 at three sites. Thirteen different phage types for *S. faecalis* were encountered in 62 different cases, phage type 6 being the most common. *S. faecium* was not frequently isolated from the cases studied.

DISCUSSION

Taxonomic classification of enterococci has been a subject of discussion. Extensive studies have been done by Deibel (9), Hartman et al. (16), and Raj and Colwell (21). Because of their low pathogenicity and the large and complex battery of tests required for their speciation, routine serotyping or biotyping in most laboratories is not done. Ciucă et al. (8) were the first to group enterococci isolated from different sources with the help of bacteriophages. Pleceas and Brandis (20) used a group of phages for rapid identification of these enterococci. It is accepted that strains belonging to *S. faecalis* group and *S. faecium* group are frequently isolated from man and animals, respectively (7,

27). Brock (5) reported that no phage attacked strains of *S. faecalis* group and of *S. faecium* group simultaneously. Our data partially confirmed these studies as, not only did we isolate four phages specific to *S. faecalis* group and six to *S. faecium* group, we also isolated seven nonspecific phages which lysed strains belonging to both of these groups.

The role of the fecal streptococci in human diseases is still unexplored. They come to medical attention as secondary invaders or opportunistic pathogens. During this study they have frequently been isolated from urogenital, anorectal, and pharyngeal regions. The same frequency of isolation of streptococci at pharyngeal, urethral, and endocervical regions and the presence of same phage types at these sites is striking. This may indicate that there is a criss-cross transmission of these microorganisms between urogenital, ano-rectal, and pharyngeal regions either by nonvenereal or most probably venereal route. The data also indicate that an individual harbors the same phage type at different sites during a given period.

Presence of similar phage types at different culture sites of the same patient indicate that the phages are specific and that phage typing of the urogenital strains is possible. Repeated phage typing of the same strains at different intervals confirmed the high reproducibility of lytic patterns. This was probably associated with the excellent stability of the phage titers in refrigerator at 4 C.

S. faecalis and nonhemolytic streptococci were isolated from 30% of 40 subjects with no history of urinary or vaginal infection and with a similar frequency from 20 women with urogenital infection (11). On the other hand, Bruce et al. (6) found a marked difference in the percentage of incidence (35%) of *S. faecalis* at vestibular, urethral, and vaginal culture sites of females with recurrent urethritis in comparison to the incidence of 12% in controls. Though the incidence of *S. faecalis* was as high as 50% in all the culture sites examined, a study in our laboratory is now in progress to evaluate with the help of bacteriophages the role of enterococci and other group D streptococci in patients of low and high risk groups for venereal diseases with and without urogenital infections.

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