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drs (Distantly Related *sic*) Gene Polymorphisms among *emm12*-Type *Streptococcus pyogenes* Isolates

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Twenty-eight *emm12*-type *Streptococcus pyogenes* isolates from patients with invasive and noninvasive infections or from asymptomatic carriers were genetically typed. Sequencing of *drs* (distantly related *sic* [streptococcal inhibitor of complement]) genes identified two novel alleles and revealed a polymorphism for *drs* similar to that of *sic*. No association was observed between the five different *drs* alleles and the five restriction patterns of the *vir* regulon for the isolates studied. These data suggest that *drs* sequencing may be useful for further differentiation of *S. pyogenes* isolates with *emm12* and identical *vir* regulon restriction patterns.

The worldwide emergence of invasive *Streptococcus pyogenes* infections since the first description of streptococcal toxic shock syndrome almost 20 years ago (4) and the persistence of these infections are still unexplained (22). Particularly, isolates with prevalent *emm* types, largely *emm1*, followed by *emm3*, *emm28*, and *emm12*, have the ability to persist in the human environment for long periods, despite the fact that the world's population has likely been exposed to these strains on numerous occasions. Results of established molecular typing methods, including restriction fragment length polymorphism pattern analysis, random amplified polymorphic DNA (RAPD) analysis, and multilocus sequence typing, have revealed only very few individual clones of invasive *emm1*-type isolates over the past 2 decades (6, 17). Moreover, identical strains have also accounted for less serious infections, such as pharyngitis, cellulitis, and even asymptomatic carriage. Whether or not the high prevalence and persistence of such strains are due to an absence of protective immunity in a significant portion of the human population or to changes in the protective epitopes of the organism remains unclear (5, 7). Previous studies have shown that subtle differences, e.g., in the surface structure of the M1 protein, render some strains resistant to immune sera. Moreover, it has been postulated that natural selection on human mucosal surfaces for variants of the *sic* gene encoding the streptococcal inhibitor of complement (SIC) contributes to the emergence or reemergence of *emm1*-type *S. pyogenes* (13). Sequencing of the highly polymorphic *sic* genes of 1,132 of *emm1*-type *S. pyogenes* isolates derived from global sources has previously shown a high level of allelic diversity, with 220 distinct genes coding for 215 SIC protein variants. This polymorphism of *sic* has been applied to genetic subtyping of *S. pyogenes* isolates with *emm1* and thus unambiguously differentiated isolates from temporally clustered invasive disease episodes (11). While the *sic* gene has previously been detected only within the *vir(mga)* regulon of all *S. pyogenes* strains harboring *emm1* and outside the *vir(mga)* regulon of *S. pyogenes* strains with *emm57* (1, 10), in a most recent study on *S. pyogenes* isolates from Japan, the presence of the *sic* gene was also reported with different frequencies for isolates harboring either *emm2*, *emm4*, *emm12*, *emm28*, *emm75*, *emm89*, *emm94*, or *emm112* (15). *drs* (distantly related *sic*) genes have been detected subsequently within the *vir(mga)* regulon of *S. pyogenes* isolates harboring *emm12* or *emm55* (10). The deduced DRS protein sequences have a leader sequence very similar to that of SIC and a high degree of similarity to one C-proximal stretch of SIC.

The purpose of this study was to determine the genetic polymorphism of *drs* genes among invasive and noninvasive *S. pyogenes* isolates with *emm12* and to evaluate *drs* as an epidemiological marker for the investigation of nosocomial infections and outbreaks.

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***S. pyogenes* isolates.** Twenty-eight epidemiologically unrelated *emm12*-type *S. pyogenes* isolates recovered from patients with invasive and noninvasive infections or from asymptomatic carriers that were referred to the National Reference Center for Streptococci in Aachen, Germany, between January 1996 and December 2000 were included in this study for further genetic typing.

***emm* gene sequencing.** *emm* typing of all 28 *S. pyogenes* isolates was performed according to the method of Podbielski et al. (19).

RAPD analysis. PCR amplification was carried out according to previously published protocols by using either (i) the 9-mer arbitrary primer H2 (5′-CCT CCC GCC ACC-3′) (20)

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TABLE 1. Results of molecular typing of *S. pyogenes* isolates harboring *emm12*

Isolate	Clinical sign(s) and/or symptom(s)[a]	Site(s) of isolation	RAPD pattern[b]		*vir* regulon pattern	*drs* allele
			A	B		
AC-1460	Cellulitis, STSS	Blood, wound	I	I	1	*drs12.04*
AC-1574	STSS, necrotizing fasciitis	Blood	I	I	1	*drs12.01*
AC-1575	Erysipelas, STSS	Blood	I	I	1	*drs12.01*
AC-1780	Nasal carriage	Nasopharynx	I	I	1	*drs12.03*
AC-1781	Nasal carriage	Nasopharynx	I	I	1	*drs12.01*
AC-1985	Osteomyelitis	Joint fluid	D*	I	2	*drs12.01*
AC-2080	SIDS	Lung tissue	I	D**	1	*drs12.03*
AC-2152	Pharyngitis, rheumatic fever	Nasopharynx	I	I	1	*drs12.01*
AC-2272	STSS	Blood	I	I	1	*drs12.03*
AC-2276	SIDS	Trachea	I	I	1	*drs12.02*
AC-2352	Pharyngitis	Nasopharynx	I	I	5	*drs12.02*
AC-2353	Pharyngitis	Nasopharynx	I	I	5	*drs12.01*
AC-2416	Bacteremia	Blood	I	I	1	*drs12.01*
AC-2417	Bacteremia	Blood	I	I	1	*drs12.01*
AC-2432	Pharyngitis	Nasopharynx	I	I	5	*drs12.02*
AC-2445	Pharyngitis	Nasopharynx	I	I	4	*drs12.02*
AC-2874	Bacteremia	Blood	I	I	1	*drs12.01*
AC-3024	Bacteremia	Blood	I	I	1	*drs12.01*
AC-3292	Surgical wound infection, abscess	Wound	I	I	1	*drs12.02*
AC-3297	Abscess, cellulitis, STSS	Wound	I	I	1	*drs12.02*
AC-3681	SIDS	Meninges, pleura	I	I	1	*drs12.01*
AC-3735	Necrotizing fasciitis, myositis	Blood, wound	I	I	1	*drs12.01*
AC-3854	Septic arthritis, necrotizing fasciitis	Joint fluid	I	I	1	*drs12.03*
AC-3954	Sepsis	Blood	I	D**	1	*drs12.01*
AC-3959	Necrotizing fasciitis	Pleural fluid	I	I	2	*drs12.01*
AC-3967	Sepsis	Blood	I	D**	1	*drs12.01*
AC-4075	Necrotizing fasciitis	Blood	I	I	1	*drs12.01*
AC-4098	Recurrent tonsillitis	Nasopharynx	I	I	3	*drs.NS488*

[a] STSS, streptococcal toxic shock syndrome; SIDS, sudden infant death syndrome.
[b] RAPD patterns were obtained by using either primer H2 (A) or primers p14 and p17 (B). I, pattern indistinguishable; D*, pattern lacking three distinct bands; D**, pattern with one aberrant band out of 15 distinct bands.

or (ii) the 10-mer arbitrary primers p14 (5′-GAT CAA GTC C-3′) and p17 (5′-GAT CTG ACA C-3′) (9).

***vir(mga)* regulon typing.** Typing of the *vir(mga)* regulon was performed by a modification of the method described by Gardiner et al. (9). To amplify the majority of the *vir(mga)* regulon, primer 5′-AAA CCG TAT CCT TTG ACG CAC TAG AGG ACA ATT TGC GAG ATT AG-3′ was used in combination with primer 5′-GAG CGC AAT GGGC AAG TTT ATC AAA TGG-3′. Cycling conditions included a preheat time of 2 min at 94°C, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 7 min. Resulting PCR products were digested in three separate reactions for 2 h by using the restriction enzymes *Hae*III, *Rsa*I, and *Bsi*YI (Roche Diagnostics, Mannheim, Germany). Fragments were separated by electrophoresis and visualized with ethidium bromide-stained agarose gel.

***sic* gene detection.** The presence of the *sic* gene was assessed by PCR with primers sic.I (5′-TAA GGA GAG GTC ACA AAC TA-3′) and sic.II (5′-TTA CGT TGC TGA TGG TGT AT-3′) as described previously (16).

***drs* gene sequencing.** For all 28 isolates, *drs* genes were sequenced on an ABI 310 automated DNA sequencer by using purified PCR products of the amplified *vir(mga)* regulon as described above and primers sicFdrs (5′-CAG CAG ATG AAG CAA GTA ATA GC-3′; positions 98 to 120 of *drs12.01*; accession number AJ 300679), sicRdrs (5′-CTT GTT TGT CAA TTT TGC TTT ACG ACC-3′; positions 861 to 835 of *drs12.01*) (10), id.for (5′-TTA AAG GAA TGG GGA ACA GCA G-3′; positions 317 to 338 of *drs12.01*), iu.rev (5′-TAT TAC TGC TGT TCC CCA TTC C-3′; positions 343 to 322 of *drs12.01*), hd.for (5′-CCT TCT GGT AAA AAC CCT C-3′; positions 535 to 553 of *drs12.01*), hu.rev (5′-GAG GGT TTT TAC CAG AAG G-3′; positions 553 to 535 of *drs12.01*), and e.for (5′-AGT AGT ATA CCA TCG CCA AG-3′; positions 745 to 764 of *drs12.01*) (2). The sequence editor DCSE, version 2.6, was used for editing, concatenation, and multiple alignments. The nucleotide sequence of *drs12.01* was used as a reference.

Results of molecular typing of the 28 *S. pyogenes* isolates are shown in Table 1. RAPD patterns were almost identical for all isolates with *emm12*. Subtyping of isolates identified five distinct restriction patterns of the *vir(mga)* regulon after digestion of the long PCR products with *Hae*III, *Bsi*YI, and *Rsa*I. The different restriction patterns of the *vir(mga)* regulon are shown in Fig. 1. No PCR product of the expected size of the presently known *sic* alleles (between 800 and 1,300 bp) was detected by PCR in any of the strains studied. Sequencing of *drs* revealed five distinct alleles. Comparison of *drs* sequences with the previously published sequences of alleles of strains NS488 and DRV1 (10) and of *drs12.01* and *drs12.02* (2) identified two novel alleles: *drs12.03* (accession no. AJ315146) and *drs12.04* (accession no. AJ315147). The *drs12.03* and *drs12.04* alleles differed from *drs12.01* by only a single nucleotide change and by three nucleotide changes, respectively, and resulted in variations in the deduced protein sequences. Figure 2 shows a partial alignment of the sequences of the new variants with

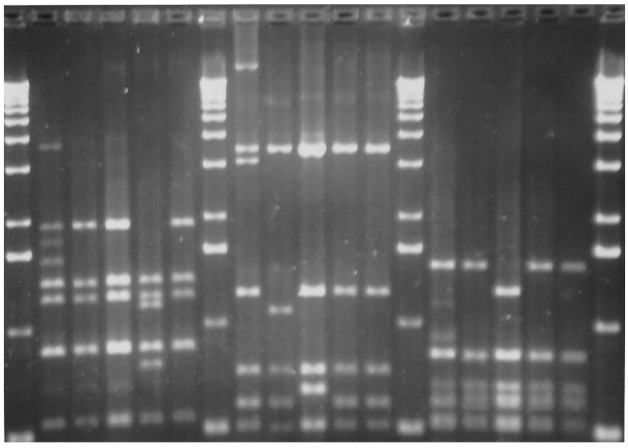

FIG. 1. Restriction fragments of the *vir(mga)* regulon after digestion of the PCR product with *Hae*III (lanes 2 to 6), *Bsi*YI (lanes 8 to 12), and *Rsa*I (lanes 14 to 18). Lanes 1, 7, 13, and 19, DNA molecular weight marker; lanes 2, 8, and 14, isolate AC-2353 (type 5); lanes 3, 9, and 15, isolate AC-2445 (type 4); lanes 4, 10, and 16, isolate AC-4098 (type 3); lanes 5, 11, and 17, isolate AC-1985 (type 2); lanes 6, 12, and 18, isolate AC-1460 (type 1).

those of the known *drs* alleles. No association was observed between individual *drs* alleles and the different restriction patterns of the *vir(mga)* regulon of the isolates studied. Except for *drs12.04*, all *drs* alleles found among invasive isolates were also found among isolates from noninvasive infections or carriers.

In contrast to findings of previous studies that have shown RAPD analysis to have additional discriminatory power over that of streptococcal M protein serotyping (20), the finding of identical RAPD patterns for almost all isolates in this study suggests that most current isolates with *emm12* are closely genetically related and supports the need for reliable and reproducible genetic subtyping methods for conducting epidemiological investigations with *S. pyogenes* isolates harboring the same *emm* type.

The fact that none of the isolates studied harbored any of the known *sic* alleles within or outside the *vir(mga)* regulon is in agreement with previous results of studies from Australia and Europe that suggest that the presence of *sic* is confined to *S. pyogenes* strains with certain *emm* types (1, 10) and contrasts with the finding of a recent study that detected *sic* in 13 of 18 *emm12*-type *S. pyogenes* isolates from Japan (15). Whether or not the high prevalence of *sic* in *emm12*-type *S. pyogenes* isolates in Japan is due to epidemiological differences or to recent recombination events needs to be studied further. Even though *drs* genes have been detected only recently in *S. pyogenes* isolates harboring *emm12* or *emm55* (10), the finding of a substantial level of genetic diversity, including two novel distinct *drs* alleles, suggests that *drs* genes in *S. pyogenes* strains with *emm12* may also exhibit a genetic polymorphism paralleling that of *sic* in *S. pyogenes* strains with *emm1*. All *drs* variants can be theoretically linked to each other by only a few molecular events, and all variants result in differences in the deduced protein sequences. This fact suggests a preference for nonsynonymous *drs* mutations over synonymous mutations, analogous to the molecular features of *sic* (11).

Previous hybridization studies and nucleotide sequence analysis from our laboratory have shown that the variations in sizes of restriction fragments of the *vir(mga)* regulon between invasive *S. pyogenes* isolates with *emm1* were largely associated with variations within the *sic* gene (3). In contrast, other stud-

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        10         20         30        120        150        340                 380       560
....|....|....|....|....|....|...  ..|....|.  ..|....|.  |....|...  ..|....|.  ....|....
ATGAAGATTAGAAATAAGATTAAAAATAGTAAA  AGCATATAT  GATTACTCT  AATATATAT  TACTATGCT  TCCCAAAAT    1 drs12.01
...............................  .........  .........  .........  ....C....  .........    2 drs12.02
.....................T.......GT.  .........  .........  .........  .........  .........    3 drs12.03
...............................  .........  .......A.  .........  .........  .........    4 drs12.04
...............................  ...------  ---------  ---......  ....C....  .........    5 drs.NS488
???????????????????????????????  ...------  ---------  ---......  .........  ....C....    6 drs.DRV1
================================================================================================================
 M  K  I  R  N  K  I  K  N  S  K     S  I  Y     D  Y  S     N  I  Y     Y  Y  A     S  Q  N      1 DRS12.01
 .  .  .  .  .  .  .  .  .  .  .     .  .  .     .  .  .     .  .  .     .  S  .     .  .  .      2 DRS12.02
 .  .  .  .  .  .  .  I  .  .  V     .  .  .     .  .  .     .  .  .     .  .  .     .  .  .      3 DRS12.03
 .  .  .  .  .  .  .  .  .  .  .     .  .  .     .  .  Y     .  .  .     .  .  .     .  .  .      4 DRS12.04
 .  .  .  .  .  .  .  .  .  .  .     .  -  -     -  -  -     -  .  .     .  S  .     .  .  .      5 DRS.NS488
 ?  ?  ?  ?  ?  ?  ?  ?  ?  ?  ?     .  -  -     -  -  -     -  .  .     .  .  .     .  P  .      6 DRS.DRV1

 610        710        720        730     750        780        940
...|....|  ....|....|....|....|....|..  ..|....|.  |....|....|.  ...|....|
AGATACTAT  GACAACCCTAATTCCCCAAGTGAACCT  AGTATACCA  GCTTCAGAGAAT  ATTAAATAG      1 drs12.01
.........  ............................  .........  ............  .........    2 drs12.02
.........  ............................  .........  ............  .........    3 drs12.03
.........  ............................  .........  ............  .........    4 drs12.04
.........  ............................  .........  ............  .........    5 drs.NS488
....T....  ....C....C.............C...  ....C....  ...C...C....  .........    6 drs.DRV1
================================================================================================
 D  T  I     D  N  P  N  S  P  S  E  P     S  I  P     A  S  E  N     I  K      1 DRS12.01
 .  .  .     .  .  .  .  .  .  .  .  .     .  .  .     .  .  .  .     .  .      2 DRS12.02
 .  .  .     .  .  .  .  .  .  .  .  .     .  .  .     .  .  .  .     .  .      3 DRS12.03
 .  .  .     .  .  .  .  .  .  .  .  .     .  .  .     .  .  .  .     .  .      4 DRS12.04
 .  .  .     .  .  .  .  .  .  .  .  .     .  .  .     .  .  .  .     .  .      5 DRS.NS488
 .  F  .     .  T  .  H  .  .  .  T  .     .  T  .     .  P  A  .     .  .      6 DRS.DRV1
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FIG. 2. Partial alignment of the nucleotide sequences of *drs* alleles *drs12.01*, *drs12.02*, *drs12.03*, *drs12.04*, *drs.NS488*, and *drs.DRV1* and of the deduced protein sequences DRS12.01, DRS12.02, DRS12.03, DRS12.04, DRS.NS488, and DRS.DRV1. The allele *drs12.01* serves as a reference.

ies have demonstrated that even though the variability of *sic* was revealed by polymorphic fragments in the *virR-emm1*-restriction fragment length polymorphism analysis, the lack of a correlation between sizes of the polymorphic restriction fragment and the *sic* allele rather suggested a size variation in an insertion segment further downstream (18). Comparison of the *vir* regulon restriction patterns in this study revealed five different patterns. The fact that no association between the *drs* polymorphism and the *vir(mga)* regulon polymorphism was observed in this study suggests that *drs* may be a valuable target for epidemiological investigations of nosocomial infections and outbreak situations caused by *S. pyogenes* with *emm12* and identical *vir(mga)* regulon patterns.

The finding that most *drs* alleles and *vir(mga)* regulon restriction patterns found among invasive isolates were also found among isolates from patients with pharyngitis or from asymptomatic carriers suggests that there is no association between the severity of disease and individual *drs* alleles. This observation provides insights into the reservoir for invasive *S. pyogenes* isolates and supports the hypothesis that the upper respiratory tract is the principal reservoir from which organisms causing invasive diseases are disseminated (8).

It has previously been shown that SIC is a secreted protein produced by M1 strains of *S. pyogenes* and that it inhibits the formation of the membrane attack complex of complement in vitro by binding to the C5b-9 complex (1). Intensive intranasal infection studies in a mouse model have shown a significantly impaired ability to colonize and persist in the upper respiratory tract for a *sic*-negative mutant compared to that for its parental strain (14). Moreover, recent studies have shown that SIC can act as an antiphagocytic effector molecule in the interior of host cells, thus providing important information on the interactions between SIC and host cells (12). Previous studies on *emm1*-type *S. pyogenes* isolates from patients with pharyngitis and treatment failure have shown that the selection of *sic* variants most likely occurs on human mucosal surfaces (2). Since epidemic waves are composed of highly heterogenous subclones based on distinct variants of *sic* (13), this diversification may contribute to an increased fitness of the isolates in the human-pathogen interactions and M1 epidemics (13). Even though previous studies have shown that DRS proteins like SIC are immunogenic in natural infections (21), it still needs to be determined in further studies whether or not SIC and DRS share common biological properties.

Nucleotide sequence accession number. Sequences of alleles *drs12.03* and *drs12.04* have been deposited under accession no. AJ315146 and AJ315147, respectively.

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