Vir Typing: A Long-PCR Typing Method for Group A Streptococci

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We have developed a new procedure (Vir typing) for typing *Streptococcus pyogenes*, by amplifying the entire 5- to 7-kb variable *vir* regulon by long PCR. The amplified DNA is then cleaved with *Hae*III and visualized by ethidium bromide fluorescence after agarose gel electrophoresis. A simple procedure for preparing DNA of sufficiently high quality from 96 samples was employed simultaneously. This DNA was also used to develop a random amplified polymorphic DNA (RAPD) procedure. The discriminatory power of the two DNA-based procedures was compared with previous methods, M typing, and multilocus enzyme electrophoresis. Both procedures were highly discriminatory, but the stoichiometric yield of restriction fragments in Vir typing allows unambiguous interpretation of results.

The application of PCR for typing microorganisms is well documented.[1] Recently Barnes reported long PCR for amplification of DNA up to 45 kb.[2] Using this technique, it is possible to amplify a specific, sufficiently large and polymorphic region of a microbial genome to identify strains. Recently, we reported long-PCR-based amplification of the ribosomal operon followed by analysis of restriction fragment length polymorphism (RFLP) to type *Haemophilus influenzae*.[3]

In this report we describe and compare two PCR-based genome typing methods for *Streptococcus pyogenes* (group A streptococci, GAS). One method, recently applied to GAS[4] is typing by random amplified polymorphic DNA (RAPD); we show that it is highly discriminatory, and we describe a protocol to handle multiple samples to obtain consistently high-quality DNA for PCR, based on the agarose-embedding procedure developed for pulsed-field electrophoresis.[5] This has resulted in highly reproducible RAPD patterns.

The second procedure is based on long PCR[2] followed by restriction endonuclease digestion and agarose gel electrophoresis. We applied this to the *vir* regulon[6] of GAS, which contains coordinately regulated[7,8] and closely linked genes.[9] The *vir* regulon consists of one or more of the following structurally related genes (collectively the *emm* gene family) arranged in tandem: genes for IgG Fc receptor (*FcrA*), for M protein (*emm*), and for IgA-binding proteins (*enn*). In addition, the *emm* gene family is flanked by the gene for C5a peptidase (*scp*A) and the regulatory gene called *virR* (*mry*)[10,6] whose function appears essential for the expression of the components of the *vir* regulon (*virR*, *scpA*, *emm* gene family). The linear order of the genes in the *vir* regulon is *virR–FcRA–emm–enn–scpA*. The rationale for choosing the *vir* regulon for PCR-based typing is as follows.

Serotypic variation of the M protein is attributable to variations in its N-proximal sequence.[11] Variations in the size of *emm* for a given serotype can also result from homologous recombination between intragenic repeats.[12] Like *emm*, *FcrA* and *enn* also exhibit polymorphism.[13,14] Moreover, architectural heterogeneity of the *vir* regulon in different isolates of *S. pyogenes* has been well documented.[9,15–17] Hence, permutations and combinations of all these components of the regulon are expected to contribute to considerable polymorphism that can be observed simply by examining restriction patterns of the PCR fragment of the regulon. As both *virR* and *scpA* genes are ubiquitous[18] among *S. pyogenes*, primers that can generate PCR fragments corresponding to this locus from all GAS isolates can be designed. This novel method (hereafter called Vir typing) is easy to perform, is sufficiently discriminatory, and could be applied to all GAS isolates tested in this study. Because the majority of isolates contained a single *vir* regulon, all restriction fragments are equimolar, avoiding ambiguities that can arise with RAPDs.

Previously, we analyzed a number of local isolates and reference strains by multilocus enzyme electrophoresis (MLEE).[19] We have now tested and analyzed these isolates and some additional local isolates by RAPD and Vir typing. On the basis of these results, we propose Vir typing as an informative typing system for GAS isolates, with RAPD being valuable where further discrimination is required.

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MATERIALS AND METHODS

Strain Selection

The 43 GAS isolates selected for analysis had been used previously in a study utilizing MLEE.[19] These strains included reference strains, strains taken from patients admitted to the Royal Darwin Hospital, and strains obtained from Aboriginal Communities of the Northern Territory.

DNA Preparation from Cells Immobilized in Agarose in a Microtiter Dish

GAS strains were grown overnight on horse blood agar plates at 37°C in 5% CO_2. One loopful of each strain was added to a round-bottomed well of a 96-well microtiter tray containing 50 μl of molten 2% low-melting-point agarose in 10 mM Tris (pH 7.5) and 1 M NaCl. The agarose plugs were then allowed to solidify on ice for 10 min. To each well of the microtiter tray was added 150 μl of EC lysis buffer (6 mM Tris at pH 7.6, 1 M NaCl, 100 mM EDTA at pH 8.0, 0.5% Brig-58, 0.2% deoxycholate, and 0.5% *N*-lauroylsarcosine) containing 1 mg/ml of lysoyme and 20 μg/ml of RNase. The trays were incubated overnight at 37°C. After incubation, lysis buffer was replaced with 150 μl of 1 mg/ml proteinase K in 0.5 M EDTA (pH 8.0), and 1% *N*-laurylsarcosine. The trays were incubated at 50°C overnight and then rinsed once in a wash solution containing 10 mM Tris (pH 7.6), and 0.1 mM EDTA. An additional 150 μl of wash solution was added, and the trays were incubated at 4°C overnight. The treatment with wash solution was repeated, and the trays were stored at −20°C until use. Prior to use, each 50 μl of GAS agarose plug was removed from the microtiter tray and placed in 450 μl of 10 mM Tris (pH 7.6) and 0.1 mM EDTA and melted at 65°C for 10 min. One microliter of this final solution was used in PCR.

RAPD Analysis

A total of 50 random oligonucleotide primers of varying lengths were screened against randomly selected GAS strains. Two of these 10-mer oligonucleotides (Operon Technology) were determined to give the best discrimination. The sequences are p14, GATCAAGTCC, and p17, GATCTGACAC. PCR amplification was performed in a DNA thermal cycler (Corbett) in 25-μl reactions containing 1 μl of DNA solution, 2.5 mM $MgCl_2$, 50 pmoles of primer, either p14 or p17, 200 μM each of dATP, dGTP, dCTP, and dTTP, 2 units of *Taq* polymerase (Bresatec), 50 mM KCl, and 10 mM Tris (pH 8.8). Forty amplification cycles were performed. Cycles 1–5 consisted of denaturation at 94°C for 30 sec, annealing at 37°C for 2 min, and extension at 72°C for 5 min. Cycles 6–40 consisted of denaturation at 94°C for 30 sec, annealing at 37°C for 60 sec, and extension at 72°C for 90 sec. After 40 cycles, 20 μl of the reaction mixture was analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide under UV light (302 nm). The gels were photographed with type 665 film (Polaroid). Films were visually compared to determine the RAPD patterns for each isolate.

Vir Typing

Forty-two strains from the group analyzed by MLEE[19]and RAPD were Vir typed. Primers for long PCR were chosen to amplify the majority of the *vir* regulon. Primer VUF is upstream from the *virR* gene, starting at nucleotide position 342 and is a combination of probes A1 and A3 used by Podbielski.[18] Primer SBR is 53 bases into the *scpA* gene (GenBank accession no. J05229). Some nucleotide mismatches were deliberately introduced in VUF and SBR primers to create restriction sites (shown in bold): VUF, 5′-AAACCGTATCTTTGACGCACT**C**GAGGACAATTTGCGAGATTAG-3′ (*Xho*I); and (SBR) 5′-AGACATGAGC**T**CAATGGCAAGTTTATCAAATGGTAATTTTTG-3′ (*Sac*I). PCR was performed essentially according to Barnes,[2] using Klentaq LA-16, PC2 buffer and 1 μl of template DNA for a 50-μl reaction. Cycling conditions were altered to include an initial 30 sec of denaturing at 94°C, followed by 25 cycles of 94°C for 10 sec, 60°C for 2 min, and 68°C for 6 min.

Five microliters of the PCR reaction was electrophoresed in a 0.8% agarose (FMC) gel to determine the quantity of DNA amplified. Without further preparation, ~0.5 μg of PCR products (8–25 μl depending on the PCR efficiency) was digested with 2 units of *Hae*III (Pharmacia) for 1 hr. The digests were electrophoresed in a 1.5% agarose gel and visualized as described in RAPD analysis. Band patterns were differentiated by visual comparison.

Statistical Methods

To assess the information content from the different methods of typing, we used the information measure from Shannon,[20] calculated as $I = -\Sigma_{i=1} r_i \log_e(r_i/n)$ (where r_i = number in i^{th} category, $\Sigma r_i = n$), with the summation over all categories for each typing method; to assess the information change when several typing methods were combined, we formed compound categories and recalculated the information measure. To assess the statistical significance of these changes we made use of the fact that differences in $2I$ follow the χ^2 distribution on 2° of freedom (d.f.).

RESULTS

Standardization and Application of RAPD

The RAPD analysis has been reported in some cases to yield irreproducible patterns, with template quality being an important parameter. As the aim of this work was to provide a basis for epidemiological studies, it was essential to develop a procedure for DNA preparation that can handle multiple samples with minimal effort and routinely provide high-quality DNA. The protocol developed for the isolation of DNA was based on a procedure for preparing DNA from organisms embedded in agarose, as this can provide intact chromosomal DNA. However, it was carried out in the wells of a microtiter dish (see Materials and Methods), enabling handling of 96 isolates simultaneously with minimal variations between samples in the treatment. The DNA recovered was of sufficient quality for routine amplification of ≥7 kb (see below) using long PCR. Most of the RAPD fragments are <2 kb.

Optimal Mg^{2+} and primer concentrations were found to be 2.5 mM and 2 μM, respectively (data not shown). Dual cycle parameters were chosen. In the first five cycles, low stringency was combined with long time for annealing and extension to allow less-than-perfect priming (see Materials and Methods). Under these conditions, the same patterns of major bands that were used in scoring RAPD types were reproduced with the

same template preparations and with independent DNA preparations from the same specimens (Fig. 1). RAPD analysis is known to produce DNA fragments of unequal intensity. In the example given in Figure 1, although three large bands and five less intense small bands were seen with primer p17, only the three large bands were used in scoring the type. The smallest of the five fainter bands was not visible in lane 1 (Fig. 1). Preparation of DNA blocks from 3-week-old cultures (held on plates) is not suitable for RAPD analysis (Fig. 1, lane 4).

Of the 43 GAS isolates belonging to 31 electrophoretic types (ETs) used in this study, RAPD analysis using p14 alone was able to distinguish 18 RAPD types, whereas p17 alone was able to distinguish 25 RAPD types. Lower discriminatory capacity with either oligonucleotide compared with ET may reflect lesser intrinsic information content, the low resolution of agarose gel electrophoresis, or the exclusion of bands that are less intense from analysis. To overcome this limitation, we combined the results from two primers in assigning the RAPD patterns to different GAS. By doing so, 35 patterns emerged, compared with 31 from ETs. Of these, 16 were reference isolates belonging to 15 different M types (Table 1). The ETs of all the reference M types were distinct from one another.[19] Their RAPD patterns were also distinct from one another. The two M3 reference isolates differ from each other with respect to a surface antigen (R antigen). They are indistinguishable by the battery of enzymes analyzed by Haase et al.,[19] but are distinguishable by RAPD.

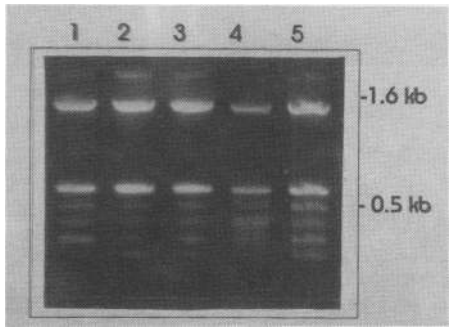

FIGURE 1 Reproducibility of RAPD. RAPD was performed using p17 as described in Materials and Methods. The products were analyzed by agarose gel electrophoresis. (Lanes *1–3*) Independent DNA preparations using the agarose-embedding procedure, made from freshly grown cultures; (lane *4*) template DNA prepared from a 3-week-old culture; (lane *5*) template DNA prepared by a standard method, which does not use agarose plugs.

TABLE 1 Summary of Typing Results

Strain	M type	Origin[a]	Date[b]	OF[c]	p14[d]	p17[e]	RAPD[f]	ET[g]	Vir[h]
PL7	M53/80	comm.	9/1/92	–	1	1	1	1	1
PL11	M53/80	comm.	9/1/92	–	1	1	1	1	1
PL12	M53/80	comm.	9/1/92	–	1	1	1	1	1
PL13	M53/80	comm.	9/1/92	–	1	1	1	1	1
PL14	M53/80	comm.	9/1/92	–	1	2	2	1	1
NS70	MNT	hosp.	5/7/92	+	9	3	3	2	2
NS14	MNT	hosp.	2/26/92	+	2	3	4	3	2
2031	M1	ref.		–	3	4	5	5	3
PL1	M5	comm.	9/1/92	–	4	5	6	6	1
PL3	M55	comm.	9/1/92	–	5	5	7	6	1
PL10	M55	comm.	9/1/92	–	5	5	7	6	1
PL6	MNT	comm.	9/1/92	–	1	6	8	7	4
NS54	MNT	hosp.	11/12/91	–	1	6	8	7	4
PL5	M55	comm.	9/1/92	–	6	7	9	9	5
PL8	M55	comm.	9/1/92	–	6	7	9	9	5
NS22	M57	hosp.	5/29/91	–	1	5	10	10	6
NS38	M55	hosp.	6/23/91	–	1	5	10	10	6
2042	M14	ref.		–	1	8	11	11	7
2033	M3	ref.		–	7	9	12	13	8
2097	M3	ref.		–	7	10	13	13	8
DOR55	M55	hosp.	5/5/80	–	8	9	14	14	9
NS81	M55	hosp.	6/26/92	–	8	9	14	15	9
2073	M53	ref.		–	5	11	15	16	10
DARL	M1	hosp.	1/13/88	–	10	26	16	17	11
PAT	M59	ref.		+	9	12	17	18	12
2317	M80	ref.		–	1	11	18	19	13
X4276	M80	hosp.*	3/12/90	–	10	13	19	19	13
2040	M12	ref.		–	9	14	20	20	14
NS100A	MNT	hosp.	11/22/92	+	17	15	21	21	15
NS17	ND	hosp.	4/10/91	–	9	16	22	23	16
2034	M4	ref.		+	9	17	23	24	17
2049	M24	ref.		–	11	18	24	25	18
2051	M26	ref.		–	12	5	25	26	19
2053	M29	ref.		–	12	14	26	26	20
PL4	MNT	comm.	9/1/92	+	18	5	27	27	21
2045	M18	ref.		–	13	5	28	28	22
PL2	M3	comm.	9/1/92	–	3	19	29	29	23
PAUL28	M28	ref.		+	9	5	30	30	24
NS49	MNT	hosp.	9/12/91	+	14	20	31	31	25
PL9	MNT	comm.	9/1/92	+	14	21	32	32	26
2035	M5	ref.		–	15	22	33	34	27
NS76	M53/80	hosp.	6/11/92	–	6	23	34	35	4
2072	M52	ref.		–	16	24	35	36	N.D.

[a](comm.) Community isolates; (hosp.) hospital isolates; (ref.) reference isolates; (hosp.*) New South Wales (Australia) hospital isolate.
[b]Date of isolation (hospital and community isolates only).
[c](OF) Opacity factor (–) Negative; (+) positive.
[d](p14) RAPD primer 14 results.
[e](p17) RAPD primer 17 results.
[f](RAPD) RAPD-typing results.
[g](ET) ET results.
[h](Vir) Vir-typing results. (N.D.) Not determined.

Vir Typing

An example of Vir typing by *Hae*III digestion of the long PCR products of 14 reference isolates belonging to distinct M serotypes is shown in Figure 2. In most cases, the yields were sufficient for a small proportion of the long-PCR mixture to be used to generate fragments that could easily be visualized by ethidium bromide staining. The yields are

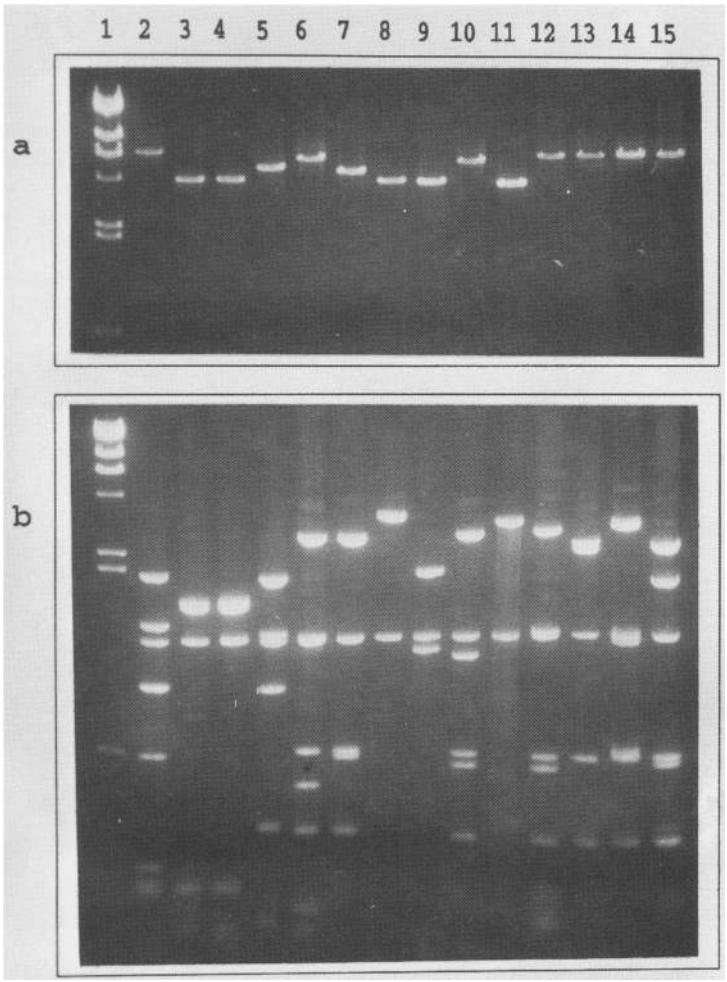

FIGURE 2 Ethidium bromide-stained agarose gels containing *vir* regulon PCR products of reference GAS isolates (*a*) and *Hae*III digestions of these PCR products (*b*). (Lane *1*) Lambda *Hin*dIII marker; (lanes *2–15*) reference isolate types M1, M3, M3(R), M5, M6, M18, M24, M26, M28, M29, M49, M53, M59, and M80, respectively.

generally much higher than those obtained by conventional PCR. As the fragments are equimolar, there is none of the ambiguity about inclusion of faint bands that can arise with RAPDs.

We analyzed the same subset of reference isolates that were used in the RAPD analysis above. Like RAPD, all 14 reference serotypes have distinct patterns by Vir typing. However, the two M3 reference isolates with different RAPD patterns were indistinguishable by Vir typing.

In addition to the reference isolates and the subset of isolates that were analyzed by MLEE, an additional 48 isolates were Vir typed. All 90 isolates tested yielded PCR products (usually between 5 and 7 kb) using the VUF and SBR primers. Digestion with *Hae*III of the PCR products yielded 40 different patterns (data not shown). Therefore, this method provides a high typability rate.

Analysis of the Local Isolates by RAPD and Vir Typing

Twenty-seven local isolates that were analyzed by MLEE were also analyzed by RAPD and Vir typing (Table 1). Fourteen of these were isolated (date 1.9.92) from a cross-sectional school survey of a community with high endemicity of GAS infection. They are represented in seven ET with one type (type 1) predominating (5/14). Both RAPD and Vir typing gave similar results; the 14 isolates represent 9 and 6 RAPD and Vir types, respectively. Interestingly, eight isolates belonging to two predominant ETs (type 1 and 6) belong to the same Vir type (type 1). This suggests that the same Vir type could occur in different genetic backgrounds within a community where the incidence of GAS infection is high.

Two Northern Territory strains, NS81 and Dor55, were isolated 12 years apart. They showed the same RAPD pattern, <5% difference in isoenzymes on MLEE analysis, and were of the same Vir type. These isolates show that genetic divergence can occur relatively slowly. This is the first observation on the stability of the *vir* regulon in nature. The Vir types of the hospital and community isolates are different from the Vir types of the reference strains; none of the latter originated in Australia. We believe that RAPD and Vir typing may together provide powerful tools to assess the relative rates of evolution by mutation and horizontal transfer of the *vir* regulon genes among GAS in endemic areas.

Information Content

For the typing results summarized in Table 1, the information contents are summarized in Table 2. It can be seen that opacity factor (OF), typing is least informative and that RAPD typing (using both p14 and p17 results) is the most informative single method. Addition of ET or Vir typing provides no additional information in the samples tested. M typing alone is less informative than RAPD, ET, or Vir typing.

DISCUSSION

Serotypic differences in GAS attributable to changes in the M protein have been well documented.[11] Although sera that were already available were adequate for typing in some parts of the world, a large proportion of isolates from other geographical locations were nontypable.[21,22] In light of the changing epidemiology of GAS infections[23,24] and continual evolution of M proteins[11,25], there is an urgent need to develop new methods of strain characterization that are generally applicable.

Several alternative methods are already available for GAS typing. Biotyping,[26] MLEE,[19,27] RFLP analysis using agarose gel electrophoresis,[28] pulsed-field gel electrophoresis,[29] and acrylamide gel electrophoresis for small fragment restriction enzyme analysis,[30] ribotyping,[26,31,32] and typing using oligonucleotides that recognize *emm* sequences[33] all have limitations.

TABLE 2 Information Content of Typing Methods as Applied to Strains in Table 1

Method	$I = \Sigma r_i \log_e(r_i/n)$[a]	i_{max}[b]	n[c]
OF	22.06	2	43
M typing	110.13	19	43
RAPD—p14 alone	109.69	18	43
RAPD—p17 alone	126.18	24	43
RAPD—p14 + p17	149.25 (144.51)[d]	35	43 (42)
Vir typing	128.80[d]	27	42[d]
Electrophoretic typing (ET)	142.07 (137.32)	33	43 (42)
ET + RAPD	150.64 (145.89)	37	43 (42)
Vir + RAPD	144.51[d]	34	42[d]

The statistical significance of adding RAPD typing to ET can be judged by taking twice the difference in information content, i.e., $2(142.07 - 150.64) = 17.14$ and referring to the χ^2 distribution on 2 d.f. ($P < 0.001$); other contrasts can be made in a similar manner.

[a](r_i) The number of isolates that fall into the i^{th} category.

[b](i_{max}) The maximum number of categories identified by the particular method for the isolates from Table 1.

[c](n) The total number of isolates tested (42 or 43).

[d]Vir typings were available for only 42 isolates; the results for other methods corresponding to the same 42 isolates are given in parenthesis.

In this paper we describe the development and comparison of two different PCR-based approaches to typing the genome of GAS. Both rely on a modified DNA peparation procedure that enables handling multiple specimens with minimal effort and produces high-quality DNA. For the development and applicability of a novel genome typing method, called Vir typing, we took advantage of sequence and architectural divergence of the constituents of the *vir* regulon of *S. pyogenes*. The typability rate by this method is high—all 90 isolates tested so far could be typed. Multiple isolates of the same Vir type were found among temporally and geographically related strains. The method is reproducible and because restriction of the PCR product yields stoichiometric amounts of the fragments, interpretation of the patterns is unambiguous.

The information content of molecular typing methods for strain identification, as summarized in Table 2, is one measure of their potential utility. For example, in epidemiological studies, it is preferable to have a typing method that has greater discrimination capacity (information content) to maximize the probability that a strain identified in one context either is or is not the same as another strain. For these purposes, RAPD or Vir-typing methods are potentially useful as well as being relatively quick and reproducible.

GAS are divided into two general classes depending on their ability to produce an OF. In addition to this phenotypic difference, the OF-positive strains and OF-negative strains can be distinguished by different alleles of *emm*[34,35] and different architecture of the *vir* regulon. The *vir* regulons of all the OF-positive strains studied to date have a single organization, whereas four possible organizations were seen in the regulons of OF-negative strains.[17] It was therefore expected that Vir typing may be less discriminatory for OF-positive strains. However, contrary to this, the nine OF-positive isolates tested had eight Vir types. These results demonstrate that the Vir typing can be applied to both classes of GAS.

Of the isolates tested, three Vir types each split into two RAPD patterns and one Vir type split into four RAPD patterns but there is no instance of a single RAPD type with different Vir types (Table 1), showing that RAPD has higher discriminatory capacity (information content) than Vir typing (Table 2). In an organism that is known to undergo changes by horizontal transfer, and in a community endemic for multiple types of GAS, RAPD analysis, which potentially has a very high discriminatory capacity, may provide important additional information for following epidemics of GAS disease and for relating GAS isolates to the nonsuppurative sequelae of poststreptococcal glomerulonephritis and acute rheumatic fever. In tropical regions where the majority of GAS isolates may be nontypable using standard M-typing sera,[21,22] and where rates of GAS related disease remain high,[36] such molecular typing will be particularly useful. Vir types measure changes in the region of the genome that encodes major virulence factors that are involved in antiphagocitic activity, attachment and colonization, and in avoiding recognition by the immune systems. We therefore believe that Vir typing is an important and informative typing method for molecular epidemiology in GAS-endemic regions.

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