MAJOR ARTICLE

Internalization-Associated Proteins among *Streptococcus pyogenes* Isolated from Asymptomatic Carriers and Children with Pharyngitis

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Sixty-two strains of *Streptococcus pyogenes* isolated from 30 asymptomatic school children and 32 children with pharyngitis were characterized to analyze the involvement of 2 fibronectin-binding proteins (F/SfbI and PrtF2/PfbpI) in *S. pyogenes* colonizing asymptomatic carriers and to determine the possible association between these proteins and the genes associated with macrolide resistance. In this study, we demonstrated that the proportion of *S. pyogenes* strains carrying the *pfbpI* gene was significantly higher among asymptomatic carriers (80%) than among children with pharyngitis (53%; $P < .05$). With regard to the proportion of *prtF1*-positive strains, no significant differences were found between the 2 groups (70% vs. 69%, for asymptomatic carriers and children with pharyngitis, respectively). Another important finding is the significant association between macrolide resistance and protein F/SfbI ($P < .001$) in both groups. These results suggest that the presence of the *pfbpI* gene can be linked to the ability of *S. pyogenes* to persist in the throat of asymptomatic carriers.

Streptococcus pyogenes that colonizes asymptomatic carriers does not cause acute disease but may enter a more quiescent state. It has been hypothesized that intracellular streptococci may represent such a reservoir [1, 2]. Fibronectin-binding proteins, such as F/SfbI, SfbII, and PrtF2/PfbpI, have a role in the adherence of *S. pyogenes* to epithelial cells [1–5]. A recent study demonstrated that the *lbp* gene encodes a laminin-binding protein (Lbp), which is one of the most important *S. pyogenes* adhesins [6].

However, it is not known whether these interactions are important to determine the group A streptococci carrier state. This prompted us to compare the involvement of *prtF1* and *pfbpI* genes associated with fibronectin-binding proteins in *S. pyogenes* that colonizes asymptomatic carriers and causes pharyngitis. Moreover, because a recent report has found an association between the presence of the *prtF1* gene and erythromycin resistance in *S. pyogenes* [5], we also investigated the correlation between the presence of these genes and phenotypes of resistance.

MATERIALS AND METHODS

Strain selection. During a 3-month period (February through April 2001), throat swabs were obtained by physicians working at the Division of Otorhinolaryngology at the University of Catania (Catania, Italy) from 30 asymptomatic school children (age, 7–10 years). The swabs had been submitted for an ear, nose, and throat examination and had yielded positive results of strep-

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Table 1. Characteristics of 30 *Streptococcus pyogenes* isolates recovered from asymptomatic carriers (group 1).

Isolate	T-type	*prtF1* gene	*pfbpl* gene	Resistance phenotype
1	W	+	+	cMLS
2	W	+	+	cMLS
3	U	+	+	cMLS
C[a]	NT	−	−	−
4	W	+	+	cMLS
5	W	+	+	cMLS
6	W	+	+	cMLS
7	T	+	+	M
8	U	−	+	EryS
9	W	+	−	cMLS
10	U	+	+	cMLS
11	NT	−	−	EryS
12	U	−	+	EryS
13	W	−	+	EryS
14	U	−	+	EryS
15	U	−	−	cMLS
16	T	+	+	M
17	U	+	+	M
18	U	−	−	cMLS
19	U	+	+	EryS
20	W	+	+	M
21	W	+	+	M
22	W	+	+	M
23	W	+	+	M
24	W	+	+	M
25	Y	+	+	EryS
26	U	+	+	EryS
27	W	+	−	EryS
28	W	−	−	EryS
29	W	−	+	EryS
30	W	+	+	M

NOTE. Eleven isolates (37%) were erythromycin susceptible (EryS), and 19 (63%) were erythromycin resistant. NT, not typed; +, present; −, absent.

[a] Negative control for *prtF1*.

tococcal rapid tests (Directigen 1-2-3 Group A Strep; Becton Dickinson; group 1). Subjects in group 1 fulfilled the following criteria: (1) no present or recent (i.e., within the 3 months before swabs were obtained) symptoms of sore throat or any other disease attributable to *S. pyogenes,* (2) no symptoms of respiratory tract infection, and (3) no receipt of antibiotic treatment within the preceding 3 months. The studied strains were isolated from pupils from different classes at 5 primary schools in Catania.

During the same period, throat swabs were obtained from children aged 8–11 years (group 2) who had pharyngitis diagnosed, who had a positive result of a streptococcal rapid test, and who had ⩾4 of the following symptoms: sore throat, fever, submandibular adenopathy, cervical lymph nodes, erythema of tonsils, and exudate on tonsils. None of the children had previously received long-term courses of antibiotic therapy or had symptoms of persistent infection.

Bacterial investigation. The 62 strains of *S. pyogenes* that

Table 2. Characteristics of 32 *Streptococcus pyogenes* isolates recovered from children with pharyngitis (group 2).

Isolate	T-type	*prtF1* gene	*pfbpl* gene	Resistance phenotype
1	T	+	−	EryS
2	T	+	+	cMLS
3	T	+	+	cMLS
C[a]	NT	−	−	−
4	T	+	+	cMLS
5	U	+	+	iMLS-A
6	T	+	+	EryS
7	T	−	−	EryS
8	U	+	−	cMLS
9	T	+	−	cMLS
10	T	−	+	EryS
11	W	+	+	M
12	U	+	+	iMLS-A
13	T	+	+	cMLS
14	W	+	+	cMLS
15	T	+	+	cMLS
16	W	+	+	M
17	U	+	−	iMLS-A
18	T	+	−	cMLS
19	T	−	−	EryS
20	W	+	−	cMLS
21	U	−	−	iMLS-B
22	T	+	+	EryS
23	W	−	+	cMLS
24	T	−	+	EryS
25	U	+	−	iMLS-C
26	U	+	+	EryS
27	U	−	−	EryS
28	T	+	−	cMLS
29	T	+	+	cMLS
30	T	−	−	cMLS
31	T	−	−	cMLS
32	U	−	−	EryS

NOTE. Ten isolates (31%) were erythromycin susceptible, and 22 (69%) were erythromycin resistant. NT, not typed; +, present; −, absent.

[a] Negative control for *prtF1*.

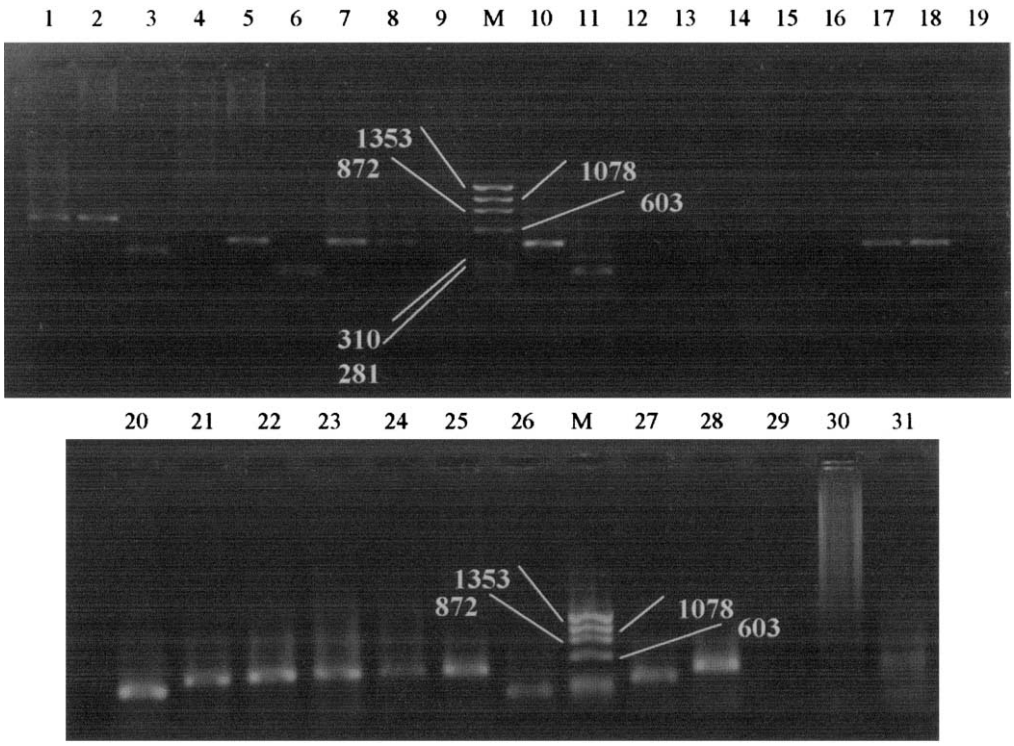

Figure 1. Detection of *prtF1* gene among 30 *Streptococcus pyogenes* strains isolated from asymptomatic carriers (group 1). PCR products in various sizes correspond to 5, 4, 3, and 2 RD2 repeat domains. C, negative control; M, marker containing DNA size (in bp).

we studied were isolated and identified in accordance with standard laboratory procedures at the Department of Microbiological and Gynecological Sciences of the University of Catania [7]. Strain identification was confirmed using a latex agglutination assay (Streptex; Wellcome). Todd-Hewitt medium (Difco) was used for routine cultures. The strains were stored in glycerol at −70°C.

Serological typing. The isolates of *S. pyogenes* were serologically typed by T-agglutination using T-typing sera organized in pools with use of SEVAC: pool T (serotypes 1, 3, 13, and B3264), pool Y (serotypes 9, 18, 22, and 23), pool W (serotypes 5, 11, 12, 27, and 44), pool U (serotypes 2, 4, 6, and 28), and pool X (serotypes 8, 14, 25, and Imp 19). The aforementioned isolated colonies were transferred to 5 mL of 0.1% Todd-Hewitt broth that contained trypsin and were incubated at 30°C overnight. The streptococcal cells were collected by centrifugation and resuspended in 0.5 mL of Todd-Hewitt broth. One drop of a 1% trypsin solution was added to the cell suspension and incubated at 37°C for 60 min. Homogeneous trypsinized cell suspensions were used as agglutinogens. Five μL of T-typing pooled sera was dropped onto a slide, and 1 loop (diameter, 3 mm) of cell suspension was mixed with a drop of serum. A reaction was considered to be positive if a marked agglutination reaction occurred within 1 min [8].

Detection of* prtF1 *and* pfbpI *genes. To detect and characterize *prtF1* and *pfbpI* genes, we used PCR with DNA primers that are complementary to the flanking region RD2 to amplify the repeat domain of these genes, which are associated with fibronectin binding [2, 3, 9] and bacterial uptake by epithelial cells. For the *prtF1* gene, the primers 5′-TTTTCAGGAAATATGGTTGAGACA-3′ (forward primer) and 5′-TCGCCGTTTCACTGAAACCACTCA-3′ (reverse primer) were used [2]; for the *pfbpI* gene, the primers, which were designed for this study, were 5′-AGGGTTCAGGTCAGGTTATTG-3′ (forward primer) and 5′-GTATTACTCTTTGGCTTATCTTT-3′ (reverse primer).

In general, PCR amplification was performed on a DNA thermal cycler (PTC-100 model; MJ Research) with use of standard concentrations, as follows: 200 μM of deoxynucleoside triphosphate, 0.2–0.4 μM concentrations of each primer, 20 μM of $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (MBI Fermentas). The cycling program consisted of 35 cycles at 94°C for 40 s, 64°C (for *prtF1;* for the *pfbpI* gene, 65°C was used) for 1 min, and 72°C for 2 min. Amplified DNA fragments were separated on 1% agarose by gel electrophoresis and visualized by ethidium bromide staining. The gels were photographed, and the bands were visually compared with results of a ϕX-174-RF DNA Hae III digest (Pharmacia). Group A streptococcal strain AP1 (*prtF1* negative) was used as negative control in each PCR experiment.

Determination of the erythromycin resistance phenotype.

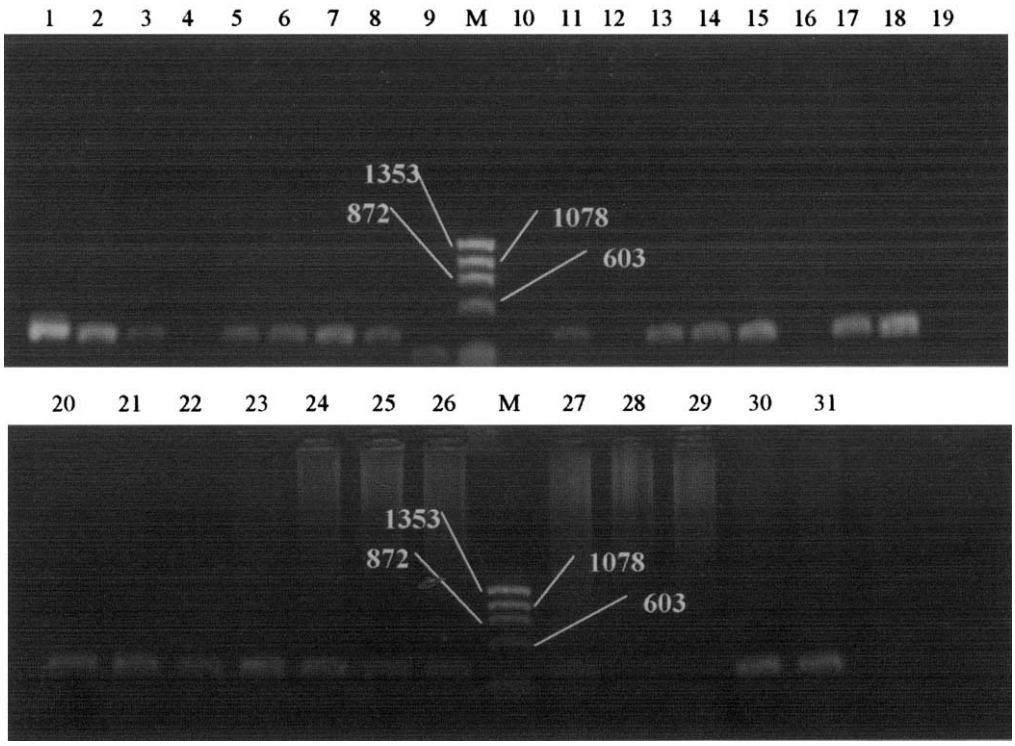

Figure 2. Detection of *pfbpI* gene among 30 *Streptococcus pyogenes* strains isolated from asymptomatic carriers (group 1). C, negative control; M, marker containing DNA size (in bp).

The MIC of erythromycin for the *S. pyogenes* isolates was determined by the broth microdilution method with use of Mueller-Hinton broth (Oxoid) supplemented with 3% lysed horse blood as the test medium. The inoculum was 5×10^5 cfu/mL.

Erythromycin-resistant isolates (MIC, $\geq$1 mg/L) [10] were characterized according to phenotype. Strains were identified as cMLS, iMLS (with subdivision of iMLS-A, iMLS-B, and iMLS-C), or M phenotypes on the basis of the results of the triple-disc test (i.e., erythromycin plus clindamycin and josamycin) [5, 11].

Statistical analysis. Proportional differences were analyzed using Fisher's exact test.

RESULTS

Tables 1 and 2 show the T-types, the presence of the *prtF1* and *pfbpI* genes, and, for erythromycin-resistant strains, the resistance phenotype in *S. pyogenes* strains isolated from asymptomatic carriers (group 1; table 1) and from subjects with pharyngitis (group 2; table 2). *S. pyogenes* isolates recovered from asymptomatic carriers included 4 pools of T-typing sera (T, W, U, and Y); 1 strain was untypable. Pool W (serotypes T5, 11, 12, 27, and 44) was the most frequently observed pool (table 1).

In group 1, *prtF1* and *pfbpI* genes were detected together in 19 (63%) of 30 isolates. The *prtF1* gene was detected alone in 2 (7%) of 30 isolates, and the *pfbpI* gene was detected alone in 5 isolates (17%). Overall, the results show that 21 strains carried the *prtF1* gene and that 24 strains carried the *pfbpI* gene. Four isolates were negative for both *prtF1* and *pfbpI* genes.

Figures 1 and 2 show the distribution of *prtF1* and *pfbpI* genes in group 1. In the RD2 region of the *prtF1* gene, the isolates recovered from subjects in group 1 had a wide range (2–5) of repeat domains (figure 1). In group 1, 19 (63%) of 30 isolates were resistant to erythromycin. The cMLS phenotype was present in 10 (53%) of 19 erythromycin-resistant strains, and the M phenotype was present in 9 (47%); iMLS phenotypes were not found (table 3). The proportion of *prtF1*-positive strains was significantly higher among erythromycin-resistant strains (17 [89%] of 19) than among erythromycin-susceptible strains (4 [36%] of 11; $P < .01$). Among the *pfbpI*-positive strains, correlation with erythromycin resistance was not significant (16 [84%] of 19 vs. 8 [73%] of 11; table 3).

S. pyogenes strains isolated from subjects with pharyngitis included 4 pools of T-typing sera (T, W, U, and Y). Pool T (serotypes 1, 3, 13, and B 2264) was the most frequently observed pool (table 2). The *prtF1* and *pfbpI* genes were detected together in 14 (44%) of 32 isolates. The *prtF1* gene was detected alone in 8 (25%) of 32 isolates, and the *pfbpI* gene was detected alone in 3 isolates (9%). Overall results show that 22 strains

Table 3. Association between presence of phenotypes of resistance and *prtF1* and *pfbpI* genes in asymptomatic carriers of *Streptococcus pyogenes* (group 1) and children with pharyngitis (group 2).

Resistance phenotype	No. of isolates	No. (%) of isolates, by gene									
		Group 1[a]					Group 2[b]				
		Total	*prtF1+/ pfbpI+*	*prtF1+/ pfbpI−*	*prtF1−/ pfbpI+*	*prtF1−/ pfbpI−*	Total	*prtF1+/ pfbpI+*	*prtF1+/ pfbpI−*	*prtF1−/ pfbpI+*	*prtF1−/ pfbpI−*
Erythromycin resistant	41	19 (63)	16 (84)	1 (5)	0	2 (11)	22 (69)	11 (50)	7 (31)	1 (5)	3 (14)
cMLS	25	10 (53)	7 (70)	1 (10)	0 (0)	2 (20)	15 (67)	7 (47)	5 (33)	1 (7)	2 (13)
iMLS-A	3	—	—	—	—	—	3 (14)	2 (67)	1 (33)	0 (0)	0 (0)
iMLS-B	1	—	—	—	—	—	1 (5)	0 (0)	0 (0)	0 (0)	1 (100)
iMLS-C	1	—	—	—	—	—	1 (5)	0 (0)	1 (100)	0 (0)	0 (0)
M	11	9 (47)	9 (100)	0 (0)	0 (0)	0 (0)	2 (9)	2 (100)	0 (0)	0 (0)	0 (0)
Erythromycin susceptible	21	11 (37)	3 (27)	1 (9)	5 (46)	2 (18)	10 (31)	3 (30)	1 (10)	2 (20)	4 (40)

NOTE. +, Present; −, absent.

[a] Thirty strains.

[b] Thirty-two strains.

carried the *prtF1* gene and 17 strains carried the *pfbpI* gene. Seven strains were negative for both *prtF1* and *pfbpI*. Figures 3 and 4 show the distribution of *prtF1* and *pfbpI* genes in group 2. Examination of PCR products for the *prtF1* gene demonstrated that the most common structures present in these strains are 4 RD2 repeat domains (figure 3).

Overall results (tables 1 and 2) showed that the proportion of strains carrying the *pfbpI* gene was significantly higher among group 1 isolates than among group 2 isolates (24 [80%] of 30 vs. 17 [53%] of 32, respectively; $P < .05$). With regard to the proportion of *prtF1*-positive strains, no significant difference was found between the 2 groups (21 [70%] of 30 isolates vs. 22 [69%] of 32 isolates; $P = .915$).

For group 2, a total of 22 (69%) of 32 isolates were resistant to erythromycin (table 3). Among the erythromycin-resistant strains, the most common phenotype was cMLS (15 [67%] of 22 isolates). The iMLS phenotype (A–C) was present in 5 isolates (23%). Only 2 strains (9%) had the M phenotype. In group 2 isolates, as in group 1 isolates, there was a statistically significative correlation between presence of the *prtF1* gene and erythromycin resistance (18 [82%] of 22 isolates were resistant to erythromycin, and 4 [40%] of 10 were susceptible to erythromycin; $P < .05$]. Among *pfbpI*-positive strains, this correlation was not significant (12 [54%] of 22 vs. 5 [50%] of 10, respectively; table 3).

Overall results (group 1 and 2) showed that the proportion of *prtF1*-positive strains was significantly higher among erythromycin-resistant strains than among erythromycin-susceptible strains (35 [85%] of 41 vs. 8 [38%] of 21, respectively; $P < .001$). The difference between the proportions of *pfbpI*-positive, erythromycin-resistant strains and *pfbpI*-positive, erythromycin-susceptible strains was not significant (28 [68%] of 41 vs. 13 [62%] of 21, respectively; $P > .05$).

DISCUSSION

Previous studies have demonstrated that fibronectin- and laminin-binding proteins of *S. pyogenes* function as adhesins and invasins [2, 4, 6, 9]. Neeman et al. [2] have hypothesized that the presence of the *prtF1* gene might be linked to the ability of a strain to persist in the throat after administration of therapy and to enter epithelial cells more efficiently. Several authors have postulated that other fibronectin-binding proteins of *S. pyogenes* can lead to a long-term persistence in the host cells [3, 4, 6]. The identification of the *prtF1* and *pfbpI* genes, the products of which have a central role in the internalization process, can allow us to confirm this idea at the molecular level. For this purpose, we examined strains of *S. pyogenes* isolated from asymptomatic carriers and from children with pharyngitis. The overall results of the present study show that the proportion of strains carrying the *prtF1* gene is equal in the 2 groups examined (70% vs. 69%) and is comparable to the proportions generally reported by other studies [4, 12, 13]. However, the proportion of *S. pyogenes* strains that carry the *prtF1* gene was found to be >80% by Facinelli et al. [5], 30% by Brandt et al. [14], and 41% by Neeman et al. [2]. Instead, the proportion of strains carrying the *pfbpI* gene is significantly higher among asymptomatic carriers of *S. pyogenes* (80%) than among children with pharyngitis (53%) ($P < .05$). On the basis of these findings, it is possible to hypothesize that *pfbpI*-bearing strains might be better colonizers of the human host and lead to throat carriage.

Another important finding is that the association between erythromycin resistance and the presence of the *prtF1* gene is statistically significant ($P < .001$). This association was significant in both groups. This result confirms the findings of Facinelli et al. [5], who suggested that, because of adjacency or

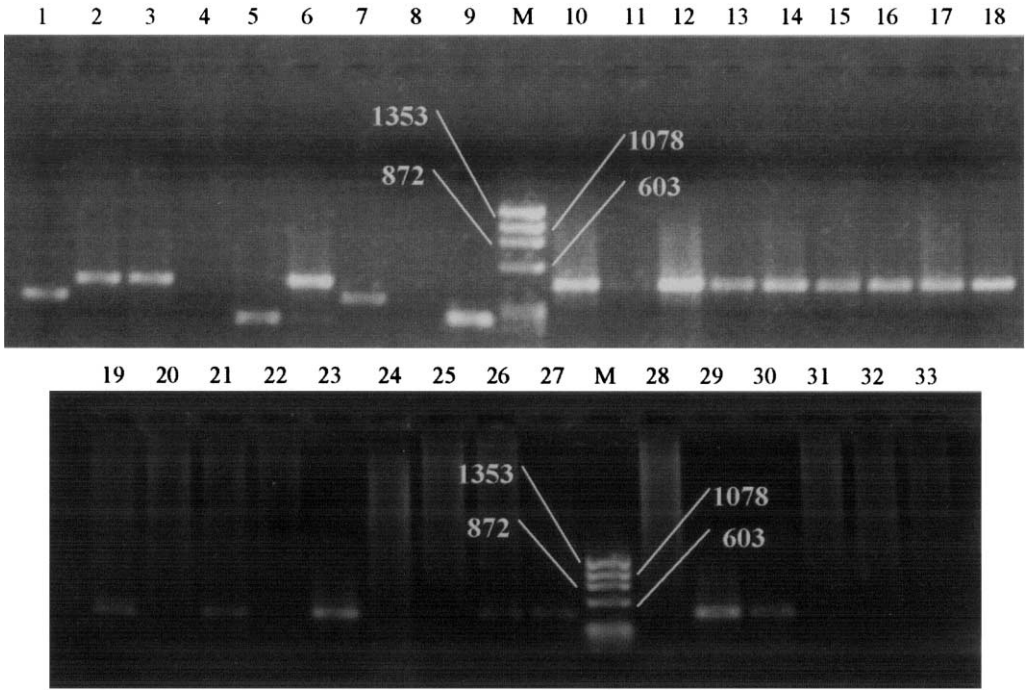

Figure 3. Detection of *prtF1* gene among 32 *Streptococcus pyogenes* strains isolated from children with pharyngitis (group 2). PCR products in various sizes correspond to 5, 4, 3, and 2 RD2 repeat domains. C, negative control; M, marker containing DNA size (in bp).

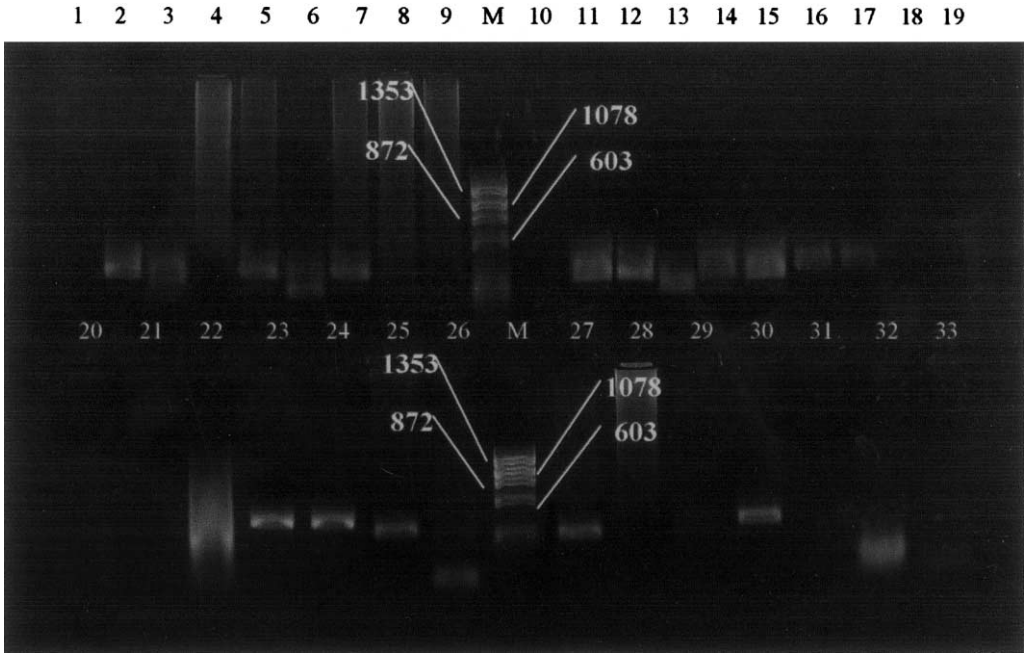

Figure 4. Detection of *pfbpl* gene among 32 *Streptococcus pyogenes* strains isolated from children with pharyngitis (group 2). C, negative control; M, marker containing DNA size (bp).

other reasons, the resistance genes and the *prtF1* gene might be transferred simultaneously on a mobile element, such as a transposon, a conjugative plasmid, or a phage.

The *prtF1* and/or *pfbpI* genes were present in 21 (84%) of 25 strains with the constitutive phenotype, in 4 (80%) of 5 inducible erythromycin-resistant strains, and in 100% of isolates with the M phenotype. Facinelli et al. [5] found the *prtF1* gene in a lower proportion (73%) of strains with the M phenotype. An interesting finding was that the iMLS phenotype was present only in strains isolated from patients with pharyngitis; in fact, it was not found in strains isolated from all carriers studied (i.e., those who had received no antibiotic treatment during the preceding 3 months). It could be possible that inducible erythromycin resistance develops after receipt of macrolide treatment. Future studies on other streptococcal genes involved in throat colonization could better elucidate the role of fibronectin- or laminin-binding proteins in the development of asymptomatic *S. pyogenes* carriage.

Acknowledgments

Rosario Musumeci performed the T-typing, performed *prtF1* and *pfbpI* detection, and phenotypically and genotypically characterized the isolates for erythromycin resistance with the collaboration of Cinzia Lo Bue. Ilaria Milazzo was involved in the clinical and bacteriological assessments and collection of strains.

Giovanna Blandino was responsible for the overall research design and data analysis and wrote the paper. Giuseppe Nicoletti and Annamaria Speciale coordinated the research on streptococci. Agostino Serra coordinated the selection of patients and the collection of specimens by physicians working at the Division of Otorhinolaryngology.

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