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emm Types, Virulence Factors, and Antibiotic Resistance of Invasive *Streptococcus pyogenes* Isolates from Italy: What Has Changed in 11 Years?▽

Roberta Creti,* Monica Imperi, Lucilla Baldassarri, Marco Pataracchia, Simona Recchia, Giovanna Alfarone, and Graziella Orefici

Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Rome, Italy

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To investigate the epidemiology and characteristics of invasive group A streptococcal (GAS) disease over 11 years in Italy, this study compared the *emm* types and the superantigen toxin genes *speA* and *speC* as well as the erythromycin, clindamycin, and tetracycline susceptibilities of 207 invasive GAS strains collected during two national enhanced surveillance periods (1994 to 1996 and 2003 to 2005) and the time between each set of surveillance periods. The present study demonstrated that *emm*1 strains were consistently responsible for about 20% of invasive GAS infections, while variations in the frequencies of the other types were noted, although the causes of most cases of invasive infections were restricted to *emm*1, *emm*3, *emm*4, *emm*6, *emm*12, and *emm*18. During the 1994 to 1996 surveillance period, an *emm*89 epidemic clone spread across the northern part of Italy. A restricted macrolide resistance phenotype-type distribution of the bacteriophage-encoded *speA* toxin as well as of macrolide resistance genes was noted over time. Indeed, the recent acquisition of macrolide resistance in previously susceptible *emm* types was observed.

Streptococcus pyogenes, or group A streptococcus (GAS), is a strictly human pathogen that infects individuals of all ages, with symptoms ranging from a carrier state to mild or acute pharyngotonsillitis and invasive disease. The organism constitutes an important cause of morbidity and mortality all over the world (10, 22, 33).

The epidemiology of severe GAS disease has been changing over the last 20 years: along with the classical suppurative and nonsuppurative forms, new manifestations such as necrotizing fasciitis and streptococcal toxic shock syndrome were described in the early 1990s, and the implementation of enhanced surveillance programs for invasive GAS infections was urged in many European countries (21).

Italy also launched an enhanced surveillance program for GAS invasive diseases in the years 1994 to 1996 in the wake of the new awareness and attention raised after the reporting of clusters of necrotizing fasciitis in England (11). The surveillance was suspended because the number of cases per year remained constant over the 3-year period (34).

A further nationwide surveillance program for invasive GAS disease was undertaken for a period of 2 years (2003 to 2005) within the Strep-EURO project on severe GAS infection in Europe, funded by the Fifth Framework Program of the European Commission's Directorate-General for Research (21).

The present investigation compared the *emm* types and the superantigen toxin genes *speA* and *speC* and the erythromycin, clindamycin, and tetracycline susceptibilities by phenotypic and molecular methods of 207 invasive GAS strains collected during the two enhanced surveillance periods (1994 to 1996 and 2003 to 2005) and in the time between each set of surveillance periods in order to study the changes in the molecular epidemiology of the strains circulating in Italy over the 11 years.

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MATERIALS AND METHODS

Strain collections. During a 2-year nationwide enhanced surveillance (from March 2003 to February 2005) as part of the Fifth Framework Strep-EURO project (21), 102 notifications of invasive infections and 89 GAS strains from patients with invasive infections were received. A case of invasive GAS disease was defined as isolation of the bacterium from a site that is normally sterile, like blood, cerebrospinal fluid, joint aspirates, pericardial and peritoneal fluids, bone, deep tissues, or abscesses, at the time of surgery or necropsy. In case of toxic shock-like syndrome, GAS strains isolated from a nonsterile site (such as the skin, throat, or vagina) were also included (1).

The study also included 79 GAS strains from patients with invasive disease received during the enhanced surveillance from 1994 to 1996 (16, 34) and 39 invasive GAS strains collected between the two enhanced surveillance periods (1997 to 2002).

Bacterial identification. The bacteria were grown overnight on 5% defibrinated sheep blood agar plates at 37°C in 5% CO_2. The bacterial strain identification was confirmed by using either the Rapid ID 32 Strep system (bioMérieux, La Balme les Grottes, France) or the Dryspot streptococcal grouping kit (Oxoid Limited, Hampshire, United Kingdom).

DNA isolation and PCR. Total DNA was prepared by a Chelex-based procedure with the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA). The isolates were investigated for the presence of the *speA* and *speC* genes by PCR, as described previously (16). A multiplex PCR for the identification of the macrolide resistance determinant *mef*(A), *erm*(B), and *erm*(A) subclass (TR) genes was performed (16). Endonuclease digestion of the *mef* amplicon with

* Corresponding author. Mailing address: Reparto di Malattie Batteriche Respiratorie e Sistemiche, Dipartimento di Malattie Infettive, Parassitarie ed Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy. Phone: 39-06-49902116. Fax: 39-06-49902886. E-mail: roberta.creti@iss.it.

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TABLE 1. Comparison of selected epidemiological aspects between the two surveillance programs

Characteristic	1994 to 1996	2003 to 2005
No. of confirmed cases/no. of strains received	97/79	102/89
Mean age (yr)	47.1 ± 23.6	39 ± 25.4[a]
% Cases in north-center-south of Italy	82-8-10	48-21-31
Mortality rate (%)[b]	24	21
Most severe clinical conditions (% of cases)		
Sepsis[c]	59	24
Necrotizing fasciitis[c]	57	16
Shock[c]	38	11

[a] Twenty-one percent pediatric (age, <12 years) cases.
[b] $P > 0.05$.
[c] $P < 0.05$.

BamHI (New England Biolabs, Beverly, MA) was carried out to ascertain the *mef*(A) allele identification (27).

The PCR conditions and primers used for the determination of the *tetM* and *tetO* genes were described previously (30).

***emm* typing.** Determination of the *emm* type was performed according to the Centers for Disease Control and Prevention protocol (7). A sequence was considered to belong to a specific *emm* gene when, over the first 160 bases of the sequence, it had 95% or greater identity with that of the reference *emm* gene.

Susceptibility test methods. Resistance to erythromycin and clindamycin was assessed phenotypically both by Etest (BIODISK, Solna, Sweden) for MIC determinations and by the Kirby-Bauer double-disk diffusion method (13) to assign the constitutive, inducible, and macrolide resistance phenotype (19). Only the strains received during the Strep-EURO survey were assayed for resistance to tetracycline, which was determined by Etest.

Statistical inference. The data were analyzed by using the STATISTICA program for Windows (StatSoft). Categorical data were compared by Fisher's exact test. Differences were considered significant when P was <0.05.

RESULTS

Comparison of the 1994 to 1996 and the 2003 to 2005 (Strep-EURO survey) enhanced surveillance programs. Both surveillance programs relied on passive notification of cases of invasive GAS infection. We promoted information about the program among hospital medical staff and through the diffusion of communications in the major national medical journals. During the most recent surveillance, an informative Web page was created at our institutional website, and an agreement was made with the National Surveillance Program on Nosocomial Infections to implement the collection of GAS strains.

The number of reported cases was slightly higher in the Strep-EURO survey than in the previous 3-year surveillance (Table 1). Participation in the survey was also more effective and homogeneous throughout the country in the Strep-EURO survey than in the previous survey. Among the 60 hospitals that adhered to the Strep-EURO initiative, 50% reported cases of severe GAS infections. The mortality rates were comparable between the two surveillance efforts (21 to 24%; $P = 0.71$). Interestingly, the mean age of the patients was lower in the Strep-EURO program, in which, contrary to the previous surveillance, pediatric cases were reported. Chickenpox was a risk factor for invasive GAS disease in only one pediatric case.

The most common clinical conditions in the former surveillance effort, such as sepsis, necrotizing fasciitis, and streptococcal shock syndrome, were significantly less represented in the Strep-EURO survey ($P < 0.05$) (Table 1).

The diverse spectrum of invasive GAS diseases in the Strep-EURO survey, either alone or in association, included abscesses with bacteremia (21%), cellulitis (10%), erysipelas with bacteremia (7%), pneumonia (7%), septic arthritis (3%), and puerperal sepsis (2%).

***emm* typing data.** The distribution of *emm* types indicated that *emm*1 strains were consistently responsible for about 20% of invasive GAS infections over the 11 years of observation, while variations in the frequencies of the other types were noted (Fig. 1). The most striking example regarded *emm*89: 25% of the invasive GAS infections in the 1994 to 1996 survey were caused by this type. Afterward, its prevalence gradually declined over time and it was responsible for only a few cases in the 2003 to 2005 survey.

In the Strep-EURO survey, *emm*1, *emm*12, *emm*3, and *emm*4 accounted for 50% of all cases. Nevertheless, 32 different types were identified in the 11 years, with fluctuations in prevalence over time. Many of the 32 types were responsible for only a few cases but were isolated throughout the study period (e.g., *emm*5, *emm*11, *emm*22, *emm*24, *emm*28, *emm*58, *emm*77, *emm*78, and *emm*118), while others (*emm*9, *emm*33, *emm*53, *emm*68, *emm*70, *emm*76, *emm*79, *emm*85, *emm*87, *emm*102, *emm*110, and *emm*114) were detected only once and have very seldom been reported in the literature to be circulating in Europe and to be involved in invasive GAS infections (35).

Among the most widespread *emm* types, *emm*1, *emm*4, *emm*12, and *emm*89 presented the same allele over the entire period of observation, while *emm*3, *emm*6, and *emm*18 presented one or two less frequent allelic variants with respect to the dominant allele (Table 2).

The *emm* types responsible for fatal cases (34/207) were *emm*1 (11 cases), *emm*3 (10 cases), *emm*4 (2 cases), *emm*5 (1 case), *emm*6 (1 case), *emm*12 (2 cases), *emm*18 (1 case), *emm*22 (1 case), *emm*77 (1 case), *emm*78 (1 case), *emm*85 (1 case), and *emm*89 (2 cases).

Susceptibility testing data. The rates of macrolide resistance of the GAS strains declined over time (26.5% in the years 1994 to 1996, 18.9% in the years 2003 to 2005; $P = 0.27$) (Fig. 2). While macrolide resistance as a result of methylation of the 23S rRNA gene mediated by the *erm*(B) gene was the predominant mechanism of resistance in the period from 1994 to 2001, the efflux-mediated mechanism of resistance by the *mef*(A) gene became prevalent among macrolide-resistant strains after 2002. An association between the *emm* types and the genetic determinants of macrolide resistance was noted during the entire period of the study, and in view of this, the dynamics of macrolide resistance could partially be explained by the decrease in the number of *emm*89 strains, which were mostly *erm*(B) positive, and to the emergence of a limited number of strains with previously susceptible *emm* types, like *emm*1 and *emm*44/61, that possessed the *mef*(A) gene (Table 3). These *emm* types were never *mef*(A) positive in a study of strains isolated from throat swabs from children from 1996 to 2001 (15, 16).

The distribution of MICs for macrolide resistance among

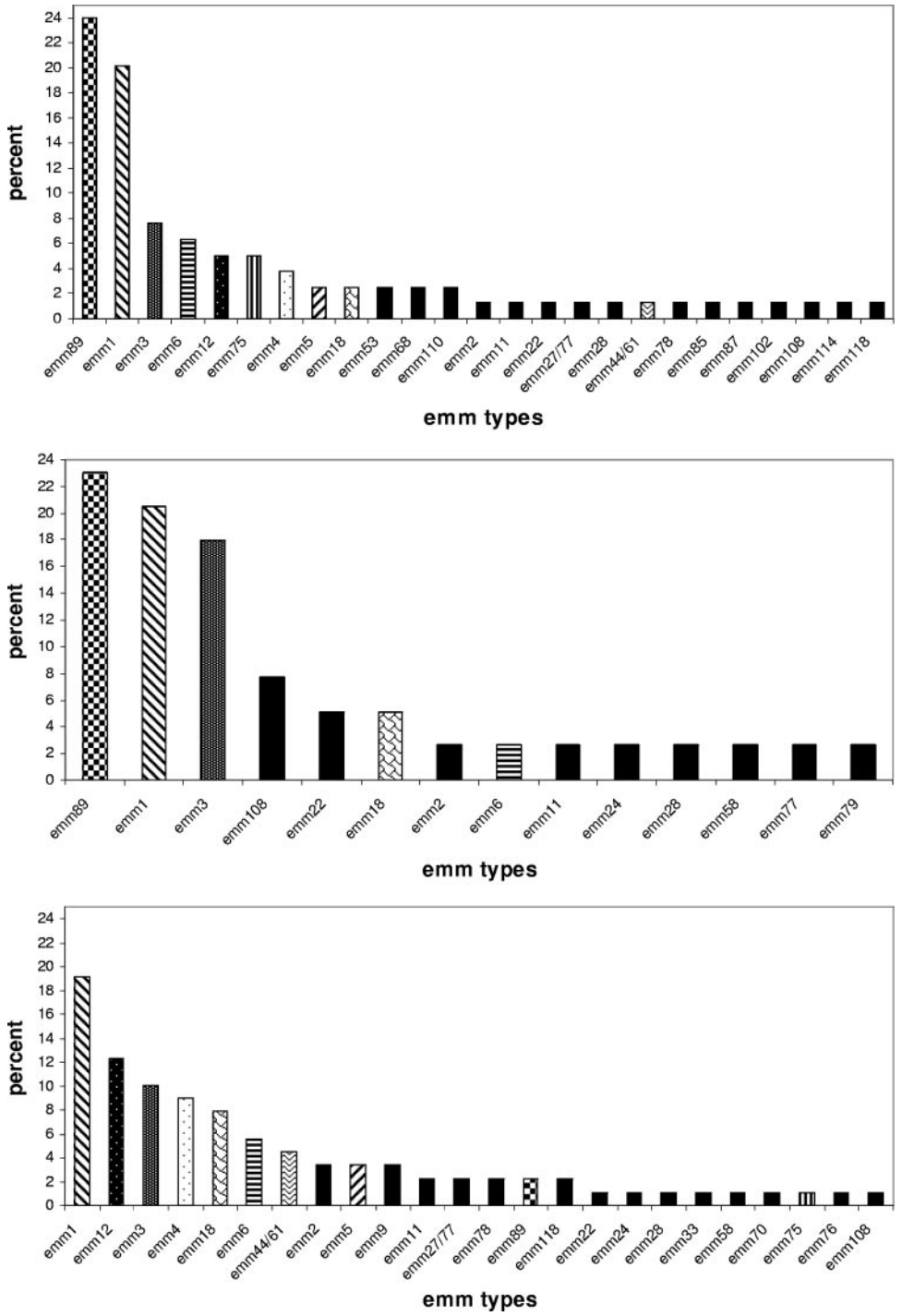

FIG. 1. Distribution of *emm* types among GAS strains isolated from patients with invasive infections during the 1994 to 1996 enhanced surveillance (top), from 1997 to 2002 (middle), and during the Strep-EURO survey (2003 to 2005) (bottom).

the strains collected during the Strep-EURO survey are represented in Fig. 3A and B and correlated well with the genotypic resistance; i.e., constitutive resistance to both erythromycin and clindamycin (CR phenotype) was associated with higher MICs and was related to the presence of the *erm*(B) gene, resistance to erythromycin and inducible resistance to clindamycin (IR phenotype) was associated with the presence of the *erm*(A) subclass (TR) gene, resistance to erythromycin was associated with lower MICs, and susceptibility to clindamycin (macrolide resistance [M] phenotype) was associated with the presence of the *mef*(A) gene.

Tetracycline resistance was assessed only for GAS strains received during the Strep-EURO survey and presented a bimodal distribution. Unlike macrolide resistance, tetracycline

TABLE 2. Description of subtypes among the most common *emm* type isolates in the 1994 to 1996 surveillance study, from 1997 to 2002, and in the Strep-EURO study

Most common *emm* type[a]	*emm* subtype(s) (%)		
	1994 to 1996	1997 to 2002	2003 to 2005
*emm*1 (16/8/17)	1.0	1.0	1.0
*emm*3 (6/7/9)	3.1	3.1	3.1 (77.8), 3.2 (22.2)
*emm*4 (3/0/8)	4.0		4.0
*emm*6 (5/1/5)	6.0	6.0	6.0 (40), 6.4 (60)
*emm*12 (4/0/11)	12.0		12.0
*emm*18 (1/2/7)	18.1	18.0	18.0 (85.7), 18.7 (14.3)
*emm*89 (19/9/2)	89.0	89.0	89.0

[a] The data in parentheses represent the numbers of isolates of each type in the 1994 to 1996 surveillance study/from 1997 to 2002/in the Strep-EURO study, respectively.

resistance was not found to be associated with particular *emm* types; all resistant strains (11 [12.3%] strains) possessed the *tetM* gene; in 3 strains it was associated both with the *tetO* gene and with macrolide resistance genetic determinants, suggesting the presence of different elements (Table 4).

Erythrogenic toxin gene distribution. The incidences of the *speA* gene were 25.3% in the 1994 to 1996 survey and 40% during the Strep-EURO study; the *speC* gene was detected in 29.1% of the isolates in the 1994 to 1996 survey and 47.2% of the isolates in the 2003 to 2005 survey, therefore showing an increasing prevalence over time. The incidences of the *speA* and *speC* genes among the invasive GAS strains collected in the period between the surveys were 46.1% and 33.3%, respectively (Fig. 4). As for the macrolide resistance genes, a restricted M phenotype-type distribution of the bacteriophage-encoded *speA* toxin was noted (*emm*1, *emm*3, *emm*18, *emm*22, *emm*24, *emm*76), while the distribution of the *speC* toxin gene was more random (data not shown).

DISCUSSION

In Italy, except for scarlet fever, severe GAS infection is not a notifiable disease. In both surveys described here, therefore, the data were collected on a voluntary basis and it was not possible to know the total number of invasive GAS infections in Italy in each year.

Considering that in the 6-year period separating the surveillance studies many fewer cases were identified and many fewer strains were collected, the ability to launch enhanced nationwide surveillance studies, even if they were based on voluntary notification, was extremely useful for the study of the dynamics of circulating strains.

The present study is the first to consider such a large number of invasive GAS strains isolated in Italy during an extended period of observation. A few studies on the prevalence over time of M-protein serotypes among invasive GAS strains in Italy are available, but only small collections of isolates were analyzed. In those studies, the majority of strains belonged to *emm* types 1, 4, and 12, followed by types 28 and 77, with *emm*1 and *emm*12 declining over time and types 3, 22, and 77 appearing more recently (25, 26).

Recent European studies that have considered a congruous number of invasive GAS isolates over a large span of time reported that similar *emm* types are involved more frequently in severe GAS disease. Given the fluctuations in the serotype distribution over time, *emm*1 isolates were the most prevalent in The Netherlands from 1994 to 2003, followed by *emm*3, *emm*89, *emm*28, *emm*12, and *emm*6 (37), as well as in Denmark, where a trend for increasing numbers of *emm*1 isolates

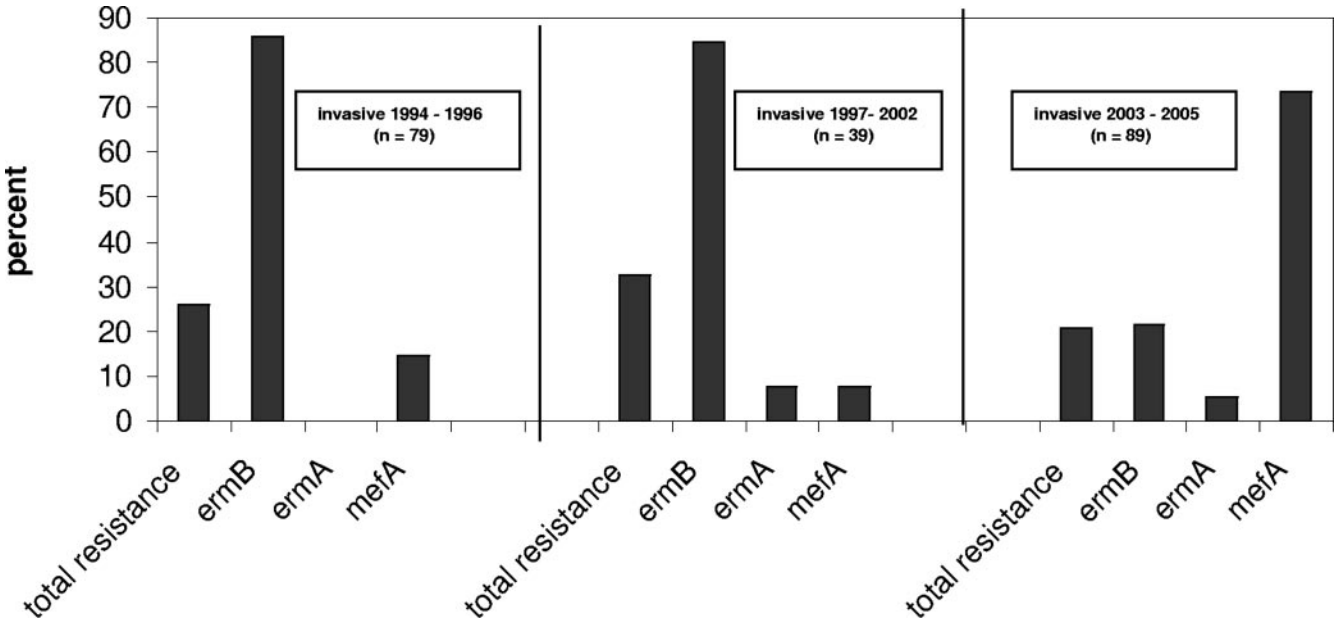

FIG. 2. Incidence of macrolide resistance and *erm*(B), *erm*(A) subclass (TR), and *mef*(A) genes among GAS strains isolated from patients with invasive infections during the 1994 to 1996 enhanced surveillance (left), from 1997 to 2002 (middle), and during the Strep-EURO survey (2003 to 2005) (right).

TABLE 3. Association among macrolide resistance genes and the *emm* types isolated during the 11 years of study[a]

emm type	No. of invasive GAS isolates (no. of isolates with the indicated associated gene)		
	1994 to 1996 surveillance study	1997 to 2002	2003 to 2005 surveillance study
1	16	8	17 **[2 *mef*(A)]**
2	1 [1 *mef*(A)]	1 [*mef*(A)]	3 [2 *mef*(A)]
3	6	7	9
4	3 [2 *mef*(A)]		8 [4 *mef*(A)]
5	2		3
6	5 [1 *erm*(B)]	1	5
9			3
11	1	1	2
12	4 [1 *erm*(B)]		11 [2 *erm*(B) **3 *mef*(A)**]
18	1	2	7 **[1 *erm*(B)]**
22	1	2 [2 *erm*(B)]	1
24		1	1
27/77	1	1 [1 *erm*(A)]	2 [1 *erm*(A)]
28	1	1	1
33			1
44/61	2		4 **[1 *mef*(A)]**
53	2		
58		1	1
68	1		
70			1
75	4		1
76			1
78	1		2
79		1	
85	1		
87	1		
89	19 [16 *erm*(B)]	9 [9 *erm*(B)]	2 [1 *erm*(B)]
102	1		
108	1	3	1
110	2		
114	1		
118	1		2
Total (n = 207)	79	39	89

[a] New associations observed in the Strep-EURO study are indicated in boldface.

in the years from 1999 to 2002 was noted (17, 18). An increasing number of M-nontypeable GAS isolates from patients with invasive diseases observed during the years from 2000 to 2002 in the United Kingdom belonged to higher M phenotype-type numbers, including *emm*89 and the newly designated *emm* types (35). A high frequency of *emm*28 and *emm*89 isolates has also been noted in recent years in other northern European countries where active nationwide surveillance programs are in place (32), and this trend has been confirmed by preliminary analysis of the data from the Strep-EURO project (20). Outside Europe, the most common *emm* types recovered from large studies of only invasive GAS infections were the M1, M3 and MPT2967 types in Canada (36); *emm*1, *emm*3, *emm*12, *emm*28, and *emm*89 in the United States (data from the Active Bacterial Core Surveillance of the Centers for Disease Control and Prevention); and M3 and M28 in Israel, where an unusually low incidence of M1 strains (1.2%) was noted (28, 29). The present investigation demonstrated that in 11 years, the causes of most cases of invasive infections in Italy were restricted to *emm*1, *emm*3, *emm*4, *emm*6, *emm*12, and *emm*18. The number of invasive infections caused by *emm*1, *emm*3, and *emm*6 was quite stable in the two surveillance periods, while the number due to *emm*12, *emm*4, and *emm*18 strains increased. The most striking differences from the data from the northern European countries were the paucity of *emm*28 strains during the entire period of observation and of *emm*89 strains in the last 7 years. It is noteworthy that during the 1994 to 1996 surveillance period, an *emm*89 epidemic clone [*speA* and *speC* negative, *erm*(B) positive] spread across the northern part of Italy and persisted until 1998. Its peak incidence was in the years 1996 and 1997.

The increased intrinsic virulence of some GAS types, particularly types M1 and M3, has been reported. The acquisition of prophages is considered the major source of genetic diversity within GAS isolates (5, 9); for example, the acquisition of *speA* appears to give to M3 isolates a selective advantage over *speA*-negative isolates (8).

A sudden increase in the numbers of invasive *emm*1 isolates with a contemporary change in the superantigen-associated repertoire (decrease of *emm*1-*speA* strains and increase of *emm*1-*speC* strains) has been interpreted to result from the possible introduction of a new *emm*1 subclone in Denmark (18). The repertoire of erythrogenic toxin genes analyzed in the present study was limited to the most often investigated *speA* and *speC* toxin gene profile, but no evident change in their prevalence among either the invasive *emm*1 or *emm*3 strains isolated was noted. In particular, *emm*1 strains always carried the *speA* gene, rarely carried the *speC* gene, and had the T1 pattern; moreover, those strains isolated during the Strep-EURO study were all of sequence type 28 (M. van Linden, personal communication). These findings are suggestive of the closeness of our *emm*1 strains to the globally disseminated clonal M1T1 strain, which is responsible for cases of highly invasive human disease and which has peculiar characteristics, such as three prophages, one of which carries the *speA* superantigen gene, and the ability to modulate the expression of selected virulence factors by phage-dependent *speB* proteolytic activity (2, 12, 14).

A more interesting finding during the recent Strep-EURO survey was the isolation of two *emm*1 strains that were macrolide resistant as a result of the acquisition of the *mef*(A) gene.

Comparison of the data from the present study with those from our previous studies indicated that over the 11-year period of observation an *emm* type presented the same subtype and repertoire of *spe* macrolide resistance genes, regardless of whether they were isolated from invasive infections or throat swab samples and regardless of whether they were responsible for either no symptoms or severe disease (16), indicating their broad spread in the community and the clonal nature of GAS strains in Italy.

The nonrandom and M phenotype-type-specific distributions of streptococcal exotoxins (in this study of *speA*) are already known, as is the lack of compelling evidence for the specific factors associated with the more severe spectrum of GAS disease (3, 4, 23, 31).

The present data confirmed the high rate of erythromycin resistance among GAS strains circulating in Italy (6, 16). Even if a slight decrease in the rate of macrolide resistance has been noted among invasive GAS strains collected in the last enhanced surveillance program, the Strep-EURO project data

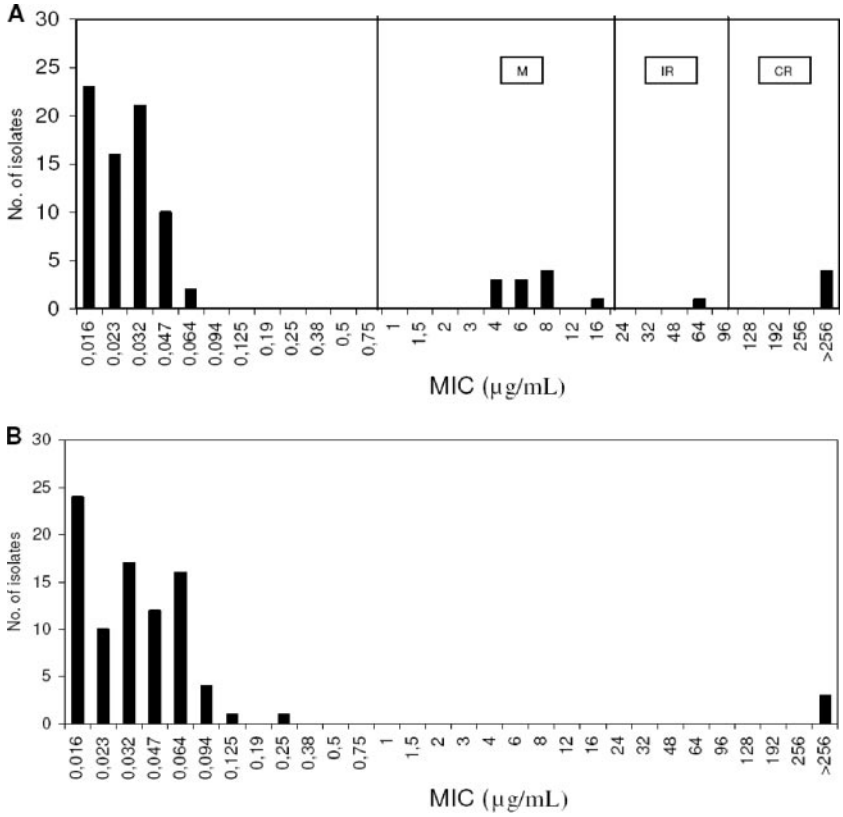

FIG. 3. Distribution of invasive GAS isolates collected during the Strep-EURO survey according to the MICs of erythromycin (A) and clindamycin (B). M, IR (inducible resistance), and CR (constitutive resistance) indicate the phenotype.

confirmed the higher rate of macrolide-resistant isolates in France and Italy compared with that in the Northern European countries participating in the project (20).

Besides different national antibiotic treatment practices, the clonal nature of the circulating strains may be the basis for the fluctuations observed among invasive GAS isolates in Italy. The epidemic *emm*89 clone always carried the *erm*(B) gene; very few other strains isolated in those years contributed to the dissemination of macrolide resistance in GAS strains from patients with invasive diseases. The dramatic reduction in the number of *emm*89-*erm*(B) isolates after 1999 and the lack of other *emm* types that successfully expressed the macrolide-lincosamide-streptogramin B resistance phenotype and that have taken its place may have contributed to the currently observed macrolide resistance rate and the prevalence of resistance mediated by *mef*(A). Indeed, the emergence of a limited number of strains possessing the *mef*(A) gene among previously susceptible *emm* types like *emm*1 and *emm*44/61 has been observed in the last 3 years.

A multivalent recombinant vaccine containing amino-terminal M-protein fragments from different serotypes of GAS plus the amino-terminal peptide fragment of Spa (surface protein antigen) has been developed and has undergone the first phase

TABLE 4. Characteristics of tetracycline-resistant invasive GAS strains isolated during the Strep-EURO survey

emm type	Tetracycline MIC (μg/ml)	*tetM*	*tetO*	Macrolide resistance mechanism
2	24	Positive	Positive	*mef*(A)
2	32	Positive	Positive	*mef*(A)
11	16	Positive	Negative	
12	16	Positive	Negative	*erm*(B)
18	48	Positive	Negative	*erm*(B)
33	32	Positive	Negative	
58	48	Positive	Negative	
70	24	Positive	Negative	
76	64	Positive	Negative	
77	12	Positive	Negative	
77	32	Positive	Positive	*erm*(A)

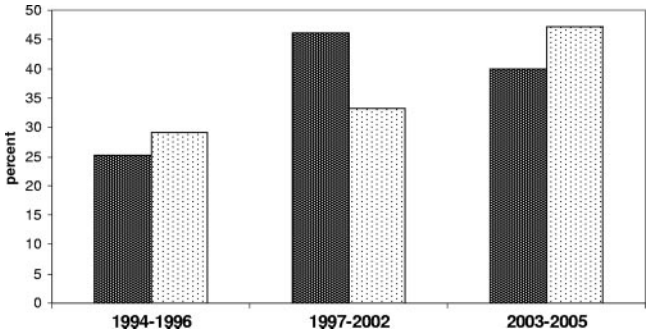

FIG. 4. Incidence of *speA* (dark gray) and *speC* (light gray) genes of invasive GAS strains isolated during the three indicated periods.

I study for its safety and immunogenicity (24). Such a vaccine would have covered 79.6% of invasive infections during the enhanced surveillance in the period from 1994 to 1996 and 75.5% of invasive infections during the Strep-EURO study.

In particular, the composition of this vaccine does not include the *emm*4 and *emm*44/61 types that, in Italy, were responsible for 3.8% and 1.3% of invasive infections in the years from 1994 to 1996, respectively, and that were responsible for an increased proportion of 9% and 4.5% of invasive infections in the more recent Strep-EURO survey, respectively. Another emerging *emm* type in our study was *emm*9, which was never detected in the past surveillance efforts and which accounted for 3.3% of invasive infections in the years from 2003 to 2005. This indicates how the continuous monitoring of the molecular epidemiology of circulating invasive GAS strains and comparison of these strains on a large scale are of crucial importance for any intervention policy and control efforts.

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