

Evolution of macrolide resistance in *Streptococcus pyogenes* over 14 years in an area of central Italy

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We evaluated temporal fluctuations in macrolide resistance rates, analysing genetic determinants of resistance and clonal evolution in a population of 2744 *S. pyogenes* isolates collected in the period 2000–2013. The total resistance rate to erythromycin of the isolates was 17.9 %. A maximum of erythromycin resistance emerged in 2000 (38.6 %), followed by a significant decrease to 5.2 % in 2012 ($P < 0.0001$). Molecular analysis revealed the presence and co-presence of known genetic resistance determinants *mefA*, *mefE*, *ermTR* and *ermB*, in line with phenotypes. PFGE analysis identified genetically related groups in 2000 and 2007–2008, mainly the MLS and M phenotypes, respectively. The most prevalent *emm* types among a representative subset of resistant isolates were *emm2*, *emm75* and *emm77*. All *emm2* and 88.2 % of the strains harbouring the *emm75* gene were only recorded in M-phenotype strains, whilst all *emm77*-positive strains had the inducible MLS phenotype. The analysed susceptible isolates showed several *emm* types partially shared with resistant ones. Our results suggest that changes in bacterial population clonality, rather than horizontal transfer of resistance determinants, plays a major epidemiological role in *S. pyogenes*. Continuous monitoring of microbiological epidemiology seems to be crucial for correct and effective management of streptococcal infections.

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INTRODUCTION

Streptococcus pyogenes is a major human Gram-positive pathogen that causes a wide range of invasive and non-invasive infections, suppurative complications and non-suppurative sequelae, including acute rheumatic fever and acute glomerulonephritis (Bisno & Stevens, 2007). Its susceptibility to penicillin remains stable, despite many years of exposure; penicillin is therefore the universally recommended treatment for *S. pyogenes* infections. Erythromycin and related antibiotics are the main alternatives in patients with penicillin allergy (Shulman *et al.*, 2012). Despite their limited indication for anti-streptococcal therapy, macrolides have often been employed as empirical therapy for respiratory tract infections due to their broad spectrum and low toxicity, in addition to their favourable

pharmacokinetic and pharmacodynamic parameters (Bisno *et al.*, 2002; Horn *et al.*, 1998). However, streptococcal resistance to macrolides and lincosamides emerged and spread worldwide until 2000 (Bingen *et al.*, 2000; Grivea *et al.*, 2006; Pérez-Trallero *et al.*, 2007). Interestingly, recent reports reveal a worldwide decrease in resistance (d'Humières *et al.*, 2012; Huang *et al.*, 2014; Montes *et al.*, 2014).

Macrolide resistance in *S. pyogenes* is caused by two main mechanisms: (i) an efflux system encoded by *mef*-class genes (M phenotype), and (ii) a target site modification encoded by *erm*-class genes (MLS phenotype). The M phenotype is associated with low-level resistance to 14- and 15-membered macrolides, whereas the MLS phenotype is associated with high-level resistance, which may be constitutive (cMLS) or inducible (iMLS) (Seppälä *et al.*, 1998; Sutcliffe *et al.*, 1996).

Understanding the epidemiology of *S. pyogenes* resistance could be useful for correct management of streptococcal infections through appropriate therapeutic approaches (Lamagni *et al.*, 2008), but data on macrolide resistance

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Abbreviations: CLSI, Clinical Laboratory Standards Institute; UPGMA, unweighted pair group method with arithmetic mean.

One supplementary figure is available with the online Supplementary Material.

rates in different European countries are still lacking. Here, we report an epidemiological and molecular study of macrolide susceptibility patterns of *S. pyogenes* isolates from an area in central Italy over a 14-year period. Our aim was to evaluate temporal fluctuations, genetic determinants and clonal evolution of macrolide resistance of *S. pyogenes* isolated in this period.

METHODS

Bacterial strains. Non-repetitive *S. pyogenes* strains ($n=2744$) were isolated from paediatric and adult patients at the Infectious Diseases Unit, Department of Medical Biotechnologies, University of Siena, Italy, from January 2000 to December 2013. They were recovered from respiratory, skin, conjunctival, genitourinary and blood sources. Isolates from sterile sites (e.g. blood) were considered invasive. All strains were community acquired, as they were isolated from outpatients or inpatients within 48 h of hospital admission. Bacterial isolation and identification were performed by standard procedures (Ruoff, 2003). Briefly, isolates were identified by colony morphology, β -haemolysis on sheep blood agar and commercial latex agglutination test (Streptococcal Grouping kit; Oxoid). Bacterial strains were preserved in Wilkins–Chalgren broth with 20 % glycerol at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Antibiotic susceptibility tests. *In vitro* susceptibility to macrolides and lincosamides was tested in all 2744 isolates. Assays were performed and the results were interpreted according to Clinical Laboratory Standards Institute (CLSI) indications (CLSI, 2012). Susceptibility to 14- and 16-membered ring macrolides (erythromycin, spiramycin) and to lincosamides (clindamycin) was determined by the Kirby–Bauer disk diffusion method on Mueller–Hinton agar with 5 % sheep blood plates (Oxoid/Thermo Scientific). Apart from the CLSI double disk indication, a triple-disk diffusion test was performed to discriminate M, cMLS and iMLS, so as to highlight any heterogeneity of inducible resistance, as described previously (Giovanetti *et al.*, 1999).

Genetic analysis. Erythromycin-resistant bacterial strains were clustered in four main representative sample groups according to resistance percentage fluctuations, including all erythromycin-resistant isolates. These were: (i) the year 2000 ($n=76$); (ii) 2002–2003 ($n=124$); (iii) 2007–2008 ($n=57$) and (iv) 2009 ($n=8$). The presence of *erm* and *mef* resistance determinants was evaluated by PCR in total clustered isolates ($n=265$). For technical reasons, it was not possible to obtain PFGE profiles for 54 erythromycin-resistant strains. A sample of 24 susceptible isolates also underwent the same PFGE and PCR analysis.

A previous study found that if two isolates belonged to the same PFGE cluster, they were likely to have the same *emm* sequence type (Carrico *et al.*, 2006). On this basis, we performed *emm* typing on a subset of 29 macrolide-resistant isolates (19 M phenotypes and 10 MLS phenotypes): one isolate representative of each distinct PFGE clone was selected in the case of more than one isolate, and when clones included isolates from different years, one isolate per year was also selected. Four resistant isolates grouped into two clones from 2003 were no longer available at the time of the *emm* typing procedures. For *emm* typing, the same sampling criteria were applied to obtain a representative subset of susceptible PFGE clones. We also added three susceptible isolates that were clonally related to macrolide-resistant ones (total $n=14$).

Genomic DNA extraction and PCR. Total genomic bacterial DNA employed as template for PCRs was obtained by boiling the bacterial

cells, as described in the CDC protocol ([http://www.cdc.gov/streplab/protocol-*emm*-type.html](http://www.cdc.gov/streplab/protocol-<i>emm</i>-type.html)). The genomic DNA was immediately used as template for PCRs or stored at $-20\text{ }^{\circ}\text{C}$ for subsequent studies. In order to amplify the *erm* and *mef* genes, primer pairs were designed as described previously (Cresti *et al.*, 2002). PCR products were analysed by agarose gel electrophoresis and ethidium bromide staining.

PFGE. Bacterial DNA was digested with *Cfr*9I (Fermentas), shown in previous studies to act on *Sma*I-resistant DNA (Bacciaglia *et al.*, 2007). Macrorestriction profile analysis was performed using dedicated software (Diversity Database, version 2.2.0; Bio-Rad) that generated unweighted pair group method with arithmetic mean (UPGMA) dendrograms, applying the band-based Dice similarity coefficient, with optimization and position tolerance settings of 1.0 and 1.5, respectively. The dendrograms revealed a PFGE cluster when a group of isolates had $\geq 80\%$ similarity (Sneath & Sokal, 1973; van Belkum *et al.*, 2007). For epidemiological purposes, clonal relationships between isolates were defined according to Tenover criteria (Tenover *et al.*, 1995): groups of isolates that were (i) genetically undistinguishable when they had the same restriction profile, or (ii) closely genetically related when they differed by fewer than four restriction fragments, were considered clones.

***emm* typing.** *emm* typing was carried out according to CDC guidelines ([http://www.cdc.gov/streplab/protocol-*emm*-type.html](http://www.cdc.gov/streplab/protocol-<i>emm</i>-type.html)). Enzymic extraction of streptococcal genomic DNA was replaced by bacterial boiling in distilled water (Tewodros & Kronvall, 2005) and PCR amplification of the *emm* gene was carried out by the CDC protocol and PCR product sequencing. Sequenced PCR products were then compared with the CDC database to retrieve the *emm* type.

Statistical analysis. Statistical analysis was performed using CDC EpiInfo (<http://www.cdc.gov/epiinfo>). Categorical variables were compared by a χ^2 test or, when appropriate, Fisher's exact test. A χ^2 test for trend was used to evaluate temporal variations. A P value of <0.05 was considered statistically significant.

RESULTS

Macrolide resistance rates

Out of 2744 strains, 2646 (96.5 %) were isolated from the respiratory tract, mostly from nasal and pharyngeal swabs; 94 (3.4 %) were isolated from skin, conjunctiva or the genitourinary tract; and four strains (0.1 %) were isolated from blood and therefore considered invasive. A total of 491 (17.9 %) erythromycin-resistant isolates were identified in the 14-year collection period. M and MLS phenotypes showed a similar distribution: 238 (8.7 %) and 253 (9.2 %) isolates, respectively.

The year-by-year temporal variations in erythromycin resistance and phenotype percentages are shown in Fig. 1. There was a significant decrease in erythromycin resistance from 38.6 % of the isolates in 2000 to 10.7 % in 2013 ($P<0.0001$). The overall decreasing trend was also significant for the single phenotypes ($P<0.0001$), but comparison of the years 2000 and 2013 highlighted a significant decrease in MLS isolates (30.4 vs 7.8 %, $P<0.0001$) associated with a non-statistically significant decrease in M strains (8.1 vs 2.9 %, $P=0.08$). Year-by-year analysis of phenotype prevalence revealed significant differences in

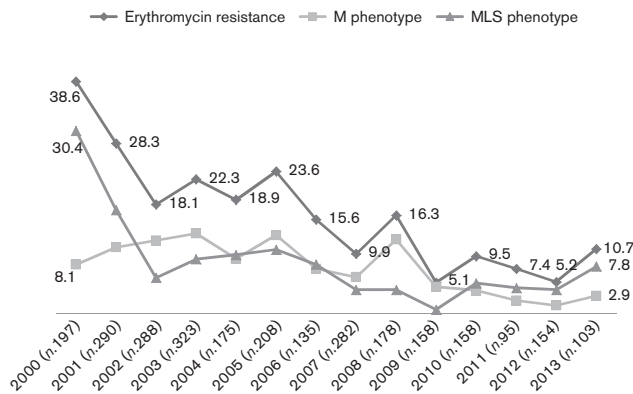


Fig. 1. Year-by-year temporal trends (%) of total erythromycin resistance and of the different phenotypes identified in the 2744 isolates of *S. pyogenes*.

the percentages of MLS isolates in the first 3 years of the study: 2000 versus 2001 (30.4 vs 17.2 %, $P < 0.0006$) and 2001 versus 2002 (17.2 vs 5.9 %, $P < 0.0001$). By contrast, a significant increase in M phenotype prevalence was only noted between 2008 (12.3 %) and 2007 (6 %, $P < 0.023$), as well as 2009 (4.4 %, $P < 0.009$).

To overcome the possible bias of annual blip variations, statistical analysis was performed grouping chemosusceptibility data by year: MLS phenotype percentage trends were in full agreement with the erythromycin resistance trend (Table 1).

Macrolide resistance genotypes

Out of 265 isolates that underwent further genetic analysis, 125 showed an MLS phenotype and 140 an M phenotype. Of the latter, 139 carried the *mefA* gene and one the *mefE* gene. Of the 125 MLS strains, 67 were iMLS and 58 cMLS phenotypes. All iMLS strains were positive for the *ermTR* gene; of these, seven isolates also carried the *ermB* gene, and one was positive for *ermTR* and *mefA*. All cMLS isolates were positive for *ermB*, three of which also carried *ermTR*.

PFGE assays

Genetic relationships between isolates were evaluated by PFGE. All but six isolates were digested by *Cfr9I*; these six showed an M phenotype and were isolated in 2009. Surprisingly, these six isolates were efficiently digested by *SmaI* enzyme. Five isolates showed an identical pattern and the other differed in two bands.

Analysis of PFGE patterns revealed 18 clones among the M-phenotype isolates and 12 clones among the MLS-phenotype isolates. M-phenotype strains were more often genetically related than MLS strains (Figs. 2 and 3). Three major

Table 1. Chemosusceptibility patterns of the 2744 *S. pyogenes* strains studied (grouped by years)

Years	Erythromycin susceptible		Erythromycin resistant		M phenotype			MLS phenotype no. (%)			P value*
	No.	%	No.	%	No. (%)	P value*	cMLS	iMLS	Total MLS		
2000–2001	487	67.6	158	32.4	48 (9.9)	<0.0001	52 (10.7)	58 (11.9)	110 (22.6)	<0.0001	
2002–2004	786	80.0	157	20.0	94 (12.0)	0.29	38 (4.8)	25 (3.2)	63 (8.0)	0.55	
2005–2007	625	84.3	98	15.7	54 (8.6)	0.05	27 (4.3)	17 (2.7)	44 (7.0)	0.01	
2008–2010	494	89.5	52	10.5	35 (7.1)	0.0003	12 (2.4)	5 (1)	17 (3.4)	0.22	
2011–2013	352	92.6	26	7.4	7 (2.0)	0.001	17 (4.8)	2 (0.6)	19 (5.4)		
Total	2744	82.1	491	17.9	238 (8.7)		146 (5.3)	107 (3.9)	253 (9.2)		

* χ^2 test comparing each previous year group.

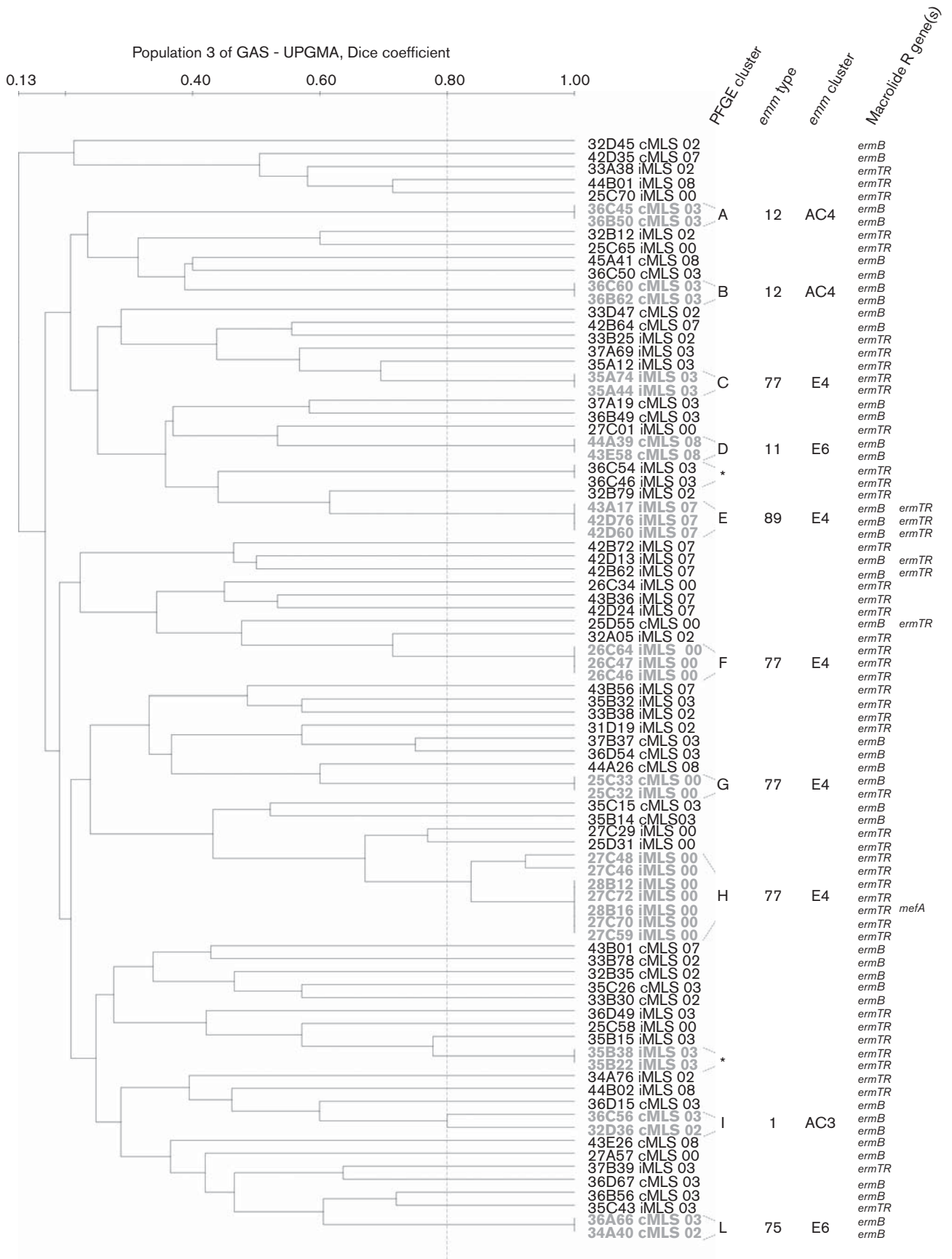


Fig. 2. Genetic relationships evaluated by PFGE in MLS phenotypes of *S. pyogenes*. Isolates in grey belong to clones subjected to *emm* typing. Clones marked with * were no longer available for *emm* typing analysis.

clones of M-phenotype isolates were observed in 2008, coinciding with the previously described peak; in one of these clones, seven isolates from 2008 were genetically indistinguishable from seven isolates of 2007 (Fig. 4).

Remarkable genetic heterogeneity was found among isolates of the years 2002–2003 (Fig. S1, available in the online Supplementary Material), whereas in 2000 and 2007–2008, clonal groups mainly consisted of MLS and M phenotypes, respectively (Figs. 4 and 5). In particular, some genetically related M strains persisted in the last two years of the study. None of the resistant clones from 2000, 2002 and 2003 persisted during the period 2007–2008.

Susceptible isolates were not usually related to macrolide-resistant strains, except for one isolate recovered in 2000, which had an identical PFGE profile to an isolate with an MLS phenotype, and two susceptible isolates recovered in 2002 with a PFGE profile identical to M-phenotype isolates.

***emm* typing**

Overall, nine *emm* types were detected among the resistant clones. The most frequent *emm* types were *emm2*, *emm75*, *emm77*, *emm12* and *emm4*, which were all recovered from larger clones (clones D and T of M-phenotype strains and clone H of MLS-phenotype strains) or were more frequent among the isolates analysed. Four different additional *emm* types (*emm1*, *emm11*, *emm42* and *emm89*) were also recovered sporadically. All but two isolates belonging to *emm* type 75 and all isolates of *emm2* and *emm4* showed an M phenotype, whereas all *emm77* type isolates belonged to the iMLS phenotype subpopulation. By contrast, susceptible isolates showed several *emm* types, four of which were common to resistant strains (*emm1*, *emm2*, *emm12* and *emm75*). The complete genotypic data of isolates that underwent *emm* typing are shown in Figs. 2 and 3 next to the isolate code.

DISCUSSION

Macrolide resistance has spread in all parts of the world, with wide geographical and temporal variations (Bingen *et al.*, 2000; Cresti *et al.*, 2002; d'Humières *et al.*, 2012; Grivea *et al.*, 2006; Huang *et al.*, 2014; Montes *et al.*, 2014; Pérez-Trallero *et al.*, 2007). The employment of macrolides as empiric anti-streptococcal therapy must therefore rely on basic resistance epidemiology data, which is lacking in European countries. In general, our findings revealed a lower rate of erythromycin resistance in *S. pyogenes* isolates than in previous Italian observations (Cornaglia *et al.*, 1998), with a significant decrease during our 14-year study period. The highest erythromycin

resistance rate, detected in the year 2000, matches a previous observation in our area in the 1990s (Cresti *et al.*, 2002). It was, however, followed by a significant decrease.

Our current chemosusceptibility data are consistent with recent observations in the USA (Villaseñor-Sierra *et al.*, 2012), other European countries (d'Humières *et al.*, 2012; Montes *et al.*, 2014) and recently, Italy (Gherardi *et al.*, 2015). A significant decrease in MLS isolates was observed, from over 30 % in 2000 to 8 % since 2007; moreover, only a few cMLS phenotype isolates were observed each year. Such a low prevalence of isolates with high-level resistance to all classes of macrolides, lincosamides and streptogramin B may be favourable for the empirical treatment of severe diseases, where clindamycin plays a major role (e.g. necrotizing fasciitis and streptococcal toxin shock syndrome) (Bisno & Stevens, 2007). In contrast, we did not observe major variations in the percentages of M-phenotype isolates; apart from sporadic peaks ('blips'), the M phenotype only seemed to contribute in a minor way to the overall erythromycin resistance trend in the study period.

The presence of known genetic resistance determinants was verified in all erythromycin-resistant isolates: *ermTR* was confirmed to be responsible for the iMLS phenotype (Varaldo *et al.*, 2009); the coexistence of two *erm* alleles (*ermB*/*ermTR*) was found in 5.6 % of all MLS isolates, although only one was phenotypically expressed, as reported previously in streptococcal and pneumococcal isolates (Cascone *et al.*, 2002; de la Pedrosa *et al.*, 2008; Giovanetti *et al.*, 2012; Silva-Costa *et al.*, 2008). In our study, certain *emm* types were specifically recovered among erythromycin-resistant isolates, and were associated with different phenotypes: *emm75*, *emm2* and *emm4* with an M phenotype and *emm77* with an iMLS phenotype. These findings are consistent with previous Italian reports (Creti *et al.*, 2005; Gherardi *et al.*, 2015; Lorino *et al.*, 2006; Zampaloni *et al.*, 2003).

Genetic and environmental factors can contribute to the spread of antibiotic resistance: horizontal transfer of resistance determinants and clonal expansion of resistant isolates often act in synergy; in this context, selective pressure caused by the use of different antibiotics can contribute to the phenomenon (Albrich *et al.*, 2004). Although our study lacks data on macrolide use during the study period, a previous report from our area (Montagnani *et al.*, 2009) revealed the absence of a significant correlation between local erythromycin resistance and overall consumption of macrolides but a significant correlation between resistance rates and a long-acting macrolide antibiotic (azithromycin). Hence, clonal analysis is a powerful tool for understanding epidemiological and temporal evolution of antibiotic resistance. In our data, PFGE analysis revealed substantial

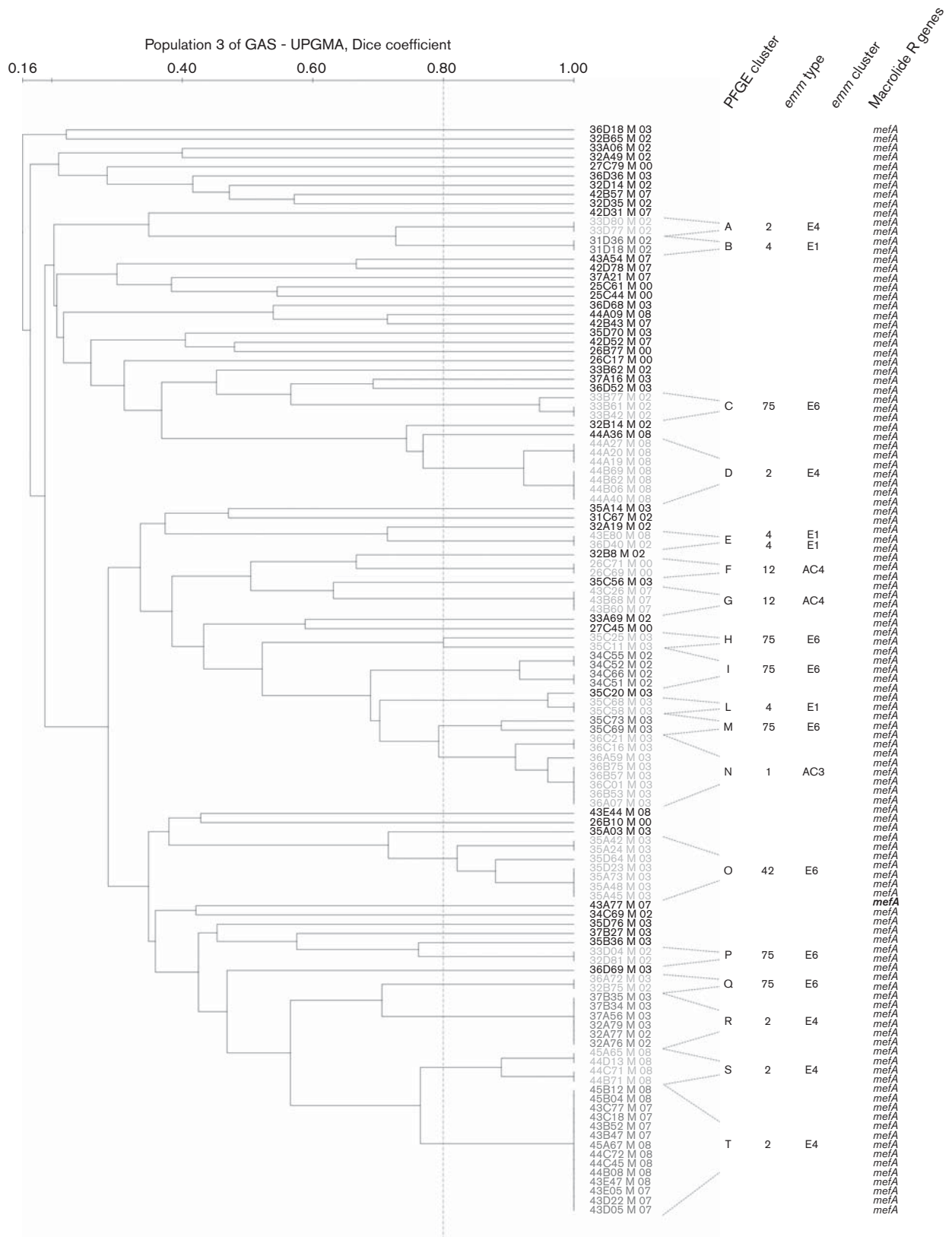


Fig. 3. Genetic relationships evaluated by PFGE in M phenotypes of *S. pyogenes*. Isolates in grey belong to clones subjected to *emm* typing.

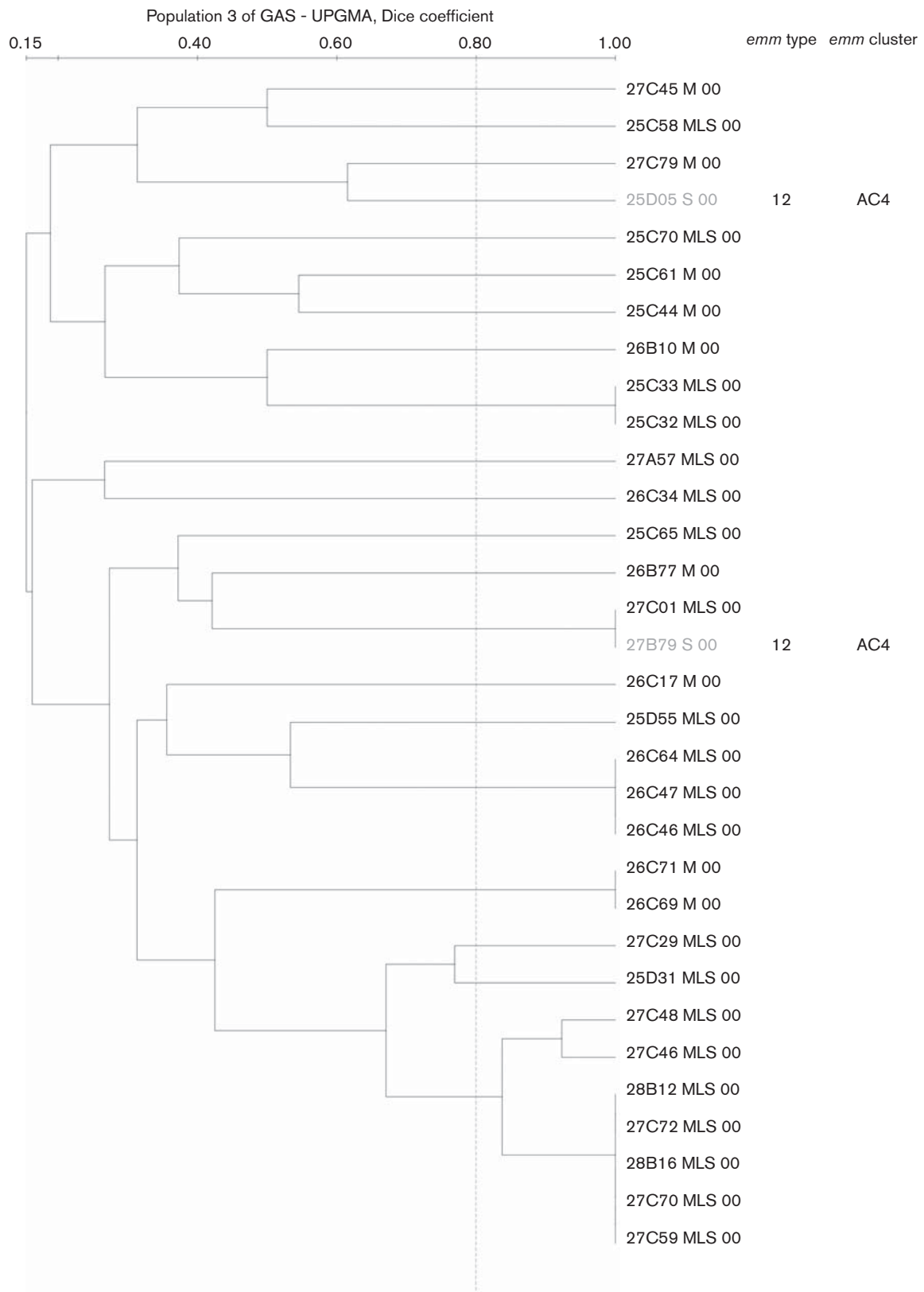


Fig. 4. Genetic relationships evaluated by PFGE in *S. pyogenes* strains studied in the years 2007–2008. Isolates in grey belong to clones subjected to *emm* typing.

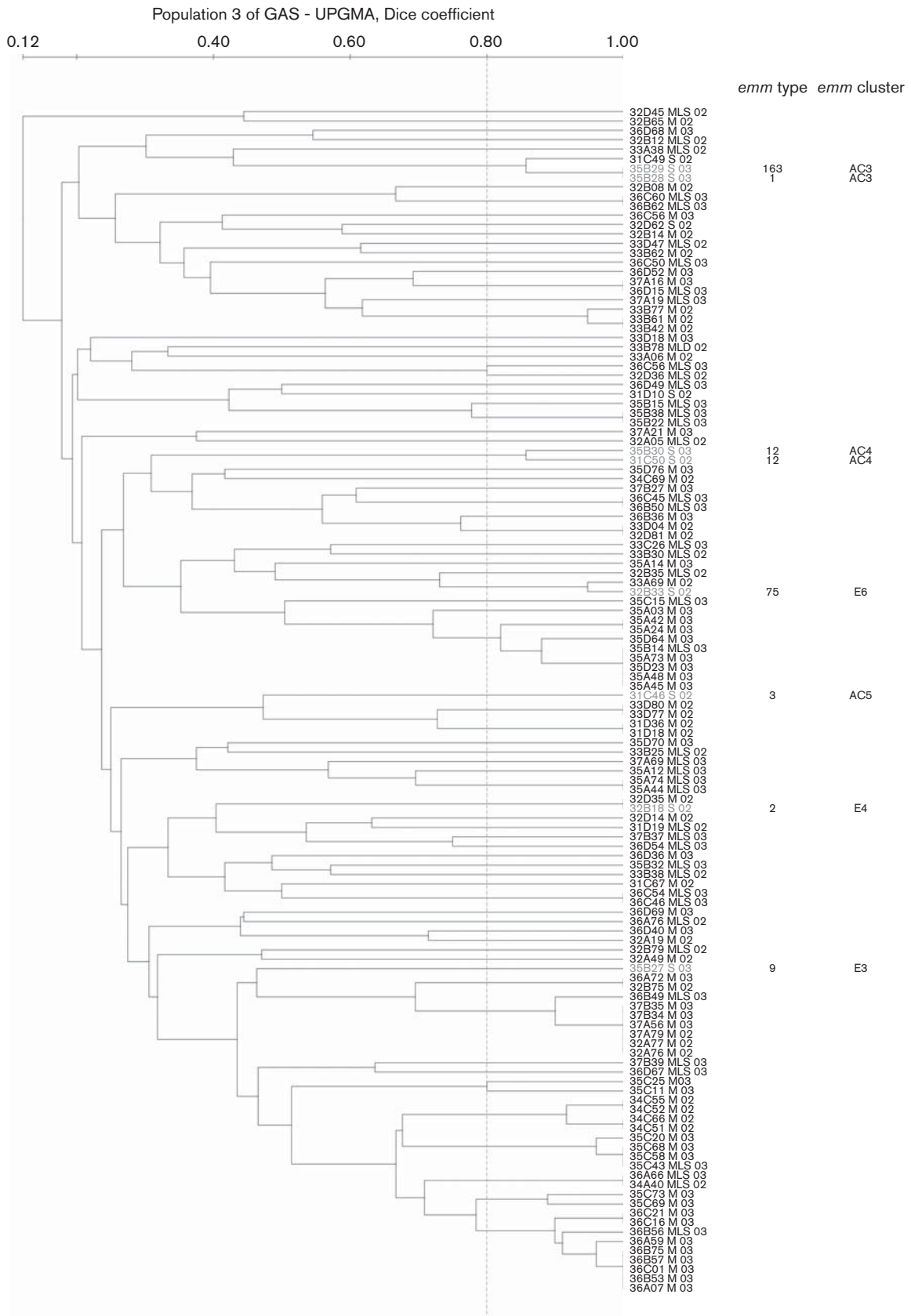


Fig. 5. Genetic relationships evaluated by PFGE in *S. pyogenes* strains studied in the year 2000. Isolates in grey belong to clones subjected to *emm* typing.

diffusion of genetically related MLS isolates in the first year of the study, when the prevalence of erythromycin resistance was highest; moreover, none of the genetically related resistant strains in 2000–2003 persisted in 2007–2008, when mainly genetically related M-phenotype isolates were found. Interestingly, a prevalence of M over MLS phenotypes was reported recently in European and non-European countries, with a similar decrease in erythromycin resistance rates (Huang *et al.*, 2014; Montes *et al.*, 2014).

Susceptible isolates revealed several *emm* types, partially shared with resistant ones.

According to our data, changes in bacterial population clonality, rather than horizontal transfer of resistance determinants, could play a major role in temporal variations in the prevalence of erythromycin resistance. A few successful clones seemed mainly associated with the spread of resistance in certain years, in line with similar epidemiological reports from other areas of Italy (Gherardi *et al.*, 2015; Zampaloni *et al.*, 2003). Data on macrolide consumption in our area during the study period is lacking, but, as antibiotic selective pressure may contribute to clonal expansion phenomena and long-acting macrolides can have a selective influence (Montagnani *et al.*, 2009), our microbiological observations suggest that changes in specific antibiotic selection pressure at the community level could be the main driver of the observed reduction in macrolide resistance. This encourages the implementation of antibiotic stewardship programme aimed at containment of the worldwide spread of macrolide resistance.

Regarding a possible bias of our study, we clarify that complete microbiological characterization of isolates (e.g. by multilocus sequence typing) was beyond the scope of our research, which was mainly aimed at supporting empirical therapeutic management of streptococcal infections. The observed temporal and geographical variations in chemosusceptibility rates suggested that continuous monitoring of microbiological epidemiology is indeed pivotal for correct and effective clinical management of streptococcal infections.

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