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M-protein gene-type distribution and hyaluronic acid capsule in group A *Streptococcus* clinical isolates in Chile: association of *emm* gene markers with *csrR* alleles

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SUMMARY

Streptococcus pyogenes causes a variety of infections because of virulence factors such as capsular hyaluronic acid and M protein. The aim of this study was to determine *emm* types and capsule phenotype in 110 isolates of *S. pyogenes* from patients with invasive (sterile sites) and non-invasive (mainly pharyngitis) infections in Chile, and the relationship between both virulence factors. The most abundant types found were *emm12*, *emm1*, *emm4* and *emm28* and their distribution was similar to that seen in Latin America and developed countries, but very different from that in Asia and Pacific Island countries. Ten of 16 *emm* types identified in pharyngeal isolates were found in sterile-site isolates, and three of nine *emm* types of sterile-site isolates occurred in pharyngeal isolates; three *emm* subtypes were novel. The amount of hyaluronic acid was significantly higher in sterile-site isolates but did not differ substantially among *emm* types. Only three isolates were markedly capsulate and two of them had mutations in the *csrR* gene that codes for a repressor of capsule synthesis genes. We found a non-random association between *emm* types and *csrR* gene alleles suggesting that horizontal gene transfer is not freely occurring in the population.

Key words: Antibiotic resistance, molecular epidemiology, *Streptococcus pyogenes*.

INTRODUCTION

Streptococcus pyogenes or group A streptococcus (GAS) is the aetiological agent of various exclusive human diseases spanning non-invasive 'strep throat' to necrotizing fasciitis or streptococcal toxic shock syndrome [1, 2]. GAS is the most common bacterial cause of acute pharyngo-tonsillitis. Moreover, infection with *S. pyogenes* may lead to the development of auto-immune disorders such as rheumatic fever and associated heart disease and acute glomerulonephritis. During the 1980s and 1990s, outbreaks of streptococcal rheumatic fever and systemic disease with severe complications were registered in many countries worldwide [2–4] and it has been suggested that this epidemiological change could have been due to an increase in the frequency of GAS types associated with severe infections [5], or an increase in strains with a high content of capsular hyaluronic acid (HA) [6]. The incidence of severe GAS diseases varies with time and geographical region, probably reflecting the susceptibility of the population to strains with

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particular virulence capabilities. GAS virulence depends on a variety of secreted and surface proteins that promote host invasion as well as evasion of the immune response. The HA capsule confers invasiveness *in vivo*, by interfering with the binding of antibodies leading to phagocytosis resistance [7]. Strains rich in HA are mucoid on agar culture and have been implicated in severe invasive infections [8]. The synthesis of HA for capsule formation is controlled by an operon which is negatively regulated by a protein, CsrR, which binds upstream of the coding region of capsule biosynthetic genes and represses their transcription [9, 10]. It has been shown that when this repressor is inactivated, HA is highly expressed and the infection produced is more invasive and severe [11]. M protein also confers resistance to phagocytosis as M-negative GAS strains are killed by this process [12]. M protein is encoded by the *emm* gene and to date more than 150 different *emm* gene types have been defined by sequence typing which due to its unambiguity and accuracy is considered the 'gold standard' for type identification of GAS [13].

M-type distribution varies across geographical regions and individual countries [14, 15]. Previous studies of genetic diversity of GAS in Chile have been limited to a reduced number of strains with specific traits and their HA content was not addressed. We aimed to determine the *emm* types and the amount of HA present in GAS strains isolated in Chile from patients with invasive (i.e. recovery of GAS from sterile sites) and non-invasive (pharyngitis) infections over a 12-year period, and to assess the relationship between *emm* type and phenotypic expression of HA with regard to virulence.

METHODS

Bacterial strains

GAS clinical isolates from multiple health centres in Chile were routinely analysed in the Clinical Microbiology Laboratory of the Pontificia Universidad Católica de Chile and stored since 1996. Non-invasive isolates were recovered mainly from throat swabs of patients with pharyngitis and invasive isolates were from diverse sterile sites of infected patients: blood, synovial fluid, bronchial secretion, abscesses and biopsy tissue. From a total of 1282 isolates from the period 1996–2007, 110 (30 invasive, 80 non-invasive) were randomly selected using computer software (Minitab) for the current study. Isolates were stored in brain-heart infusion broth (Merck, Germany) supplemented with 5% sheep blood and subcultured on Columbia blood agar (bioMérieux, France).

emm gene typing

Strains were sequenced according to Centers for Disease Control and Prevention (CDC) protocol [16]. Briefly, DNA was extracted from the isolates using the Qiagen DNA extraction kit (Qiagen, Germany), amplified by PCR with standard primers [17] and following column purification were sequenced using the M1a primer [17]. The sequence obtained was compared with the CDC database and assigned an *emm* type [16]. In accordance with CDC criteria, a new *emm* type was defined if a sequence identity of $<92\%$ with the reference type (identified as subtype .0) was observed in the 90 nucleotides encoding the first 30 amino acids of the processed M protein. Subtypes were assigned based on any change in the sequence of the first 50 amino acids of the processed M protein. All sequences found to be different from the reference sequence were searched for in the GenBank database using the BLAST program to confirm their novelty.

csrR gene sequencing

A product of 714 bp was amplified with the following primers: CsrF: GGGTTGGTATAAATGACAAAG and CsrR: GATTTTCCATATGACTTATTTC and this was sequenced with CsrF primer. Sequences were compared to a wild-type *csrR* gene sequence (accession no. AB513958.1) using the ClustalW programme. The dendrogram was produced using UPGMA cluster analysis [18] and the maximum composite likelihood method [19] with Mega4 software [20]. All sequences that showed variations with respect to the wild-type sequence were searched for in the GenBank database to confirm their novelty.

Mucoid phenotype and capsular HA content

The expression of capsule by isolates was determined by visual inspection of colonies grown on Columbia blood agar for 24 h with 5% of CO_2. Mucoid (capsulate) isolates were characterized by a brilliant halo while non-mucoid strains grew with a matt appearance. For the quantification of capsular HA the

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Stains-all method was used [21]. Briefly, isolates were cultivated for about 4–6 h in Todd–Hewitt broth (Becton-Dickinson, France) at 37 °C to an optical density (600 nm) of 0·6–0·8. After plating serial dilutions of this broth on Columbia blood agar for colony-forming unit (c.f.u.) quantification, bacteria were centrifuged at 8000 ***g***, washed with distilled water and incubated at room temperature with chloroform for 1 h to release capsular HA. After centrifugation at 12 400 ***g*** for 5 min, 100 μl of the supernatant were added to 1 ml of freshly prepared Stains-all reagent (Sigma, USA) and the absorbance was measured at 640 nm. To quantify HA content, a calibration curve was prepared with known concentrations of a reference HA (EMD Biosciences, USA) (0·005, 0·01, 0·02, 0·03 μg/μl). The amount of HA was expressed as femtograms per c.f.u. (fg/c.f.u.). Reference control strains of mucoid (87–282 [22]) and non-mucoid (771cap$^-$ [8]) were kindly provided by Dr Michael Wessels (Medical School, Harvard University, USA).

Erythromycin susceptibility testing

Erythromycin susceptibility was determined for all isolates by the agar diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2006) [23]. Reference strains of known antibiotic susceptibility were used as controls and inhibition zone diameters were measured and interpreted according to CLSI criteria [24].

Statistical analysis

One-way analysis of variance (ANOVA), Student's unpaired two-tailed *t* test and χ^2 analysis were performed with GraphPad Prism 5.0 software (GraphPad, USA). To measure the association between *csrR* gene and *emm* type, 2×2 contingency tables were used. GraphPad software was used to calculate statistical significance using χ^2 analysis with Yates' correction for low sample size.

RESULTS

emm-type distribution among GAS clinical isolates

Nineteen different *emm* types were identified and the most frequent were *emm12* (19·1%), *emm1* (14·5%), *emm4* (11·8%), and *emm28* (8·2%); the six predominant *emm* types accounted for 67% of the total isolates (Table 1). Sixteen different *emm* types were found among pharyngeal isolates, 10 of which were absent from sterile-site isolates (*emm4, emm2, emm77, emm9, emm94, emm78, emm87, emm3, emm73, emm89*). By contrast, the 30 sterile-site isolates were represented by nine different *emm* types, three of which (*emm11, emm48, emm81*) were absent from pharyngeal isolates. Type *emm4* was frequent among pharyngeal isolates but was not found in sterile sites. Similarly, type *emm75* accounted for 13·3% of sterile-sites isolates but only 2·5% of pharyngeal isolates.

Of the 19 *emm* types identified, all but five contained representatives of the reference type (.0) but four types (*emm1, emm2, emm6, emm12*) were heterogeneous in subtype (Table 1); *emm12* comprised five subtypes with the reference type 12·0 being the most predominant, in contrast with *emm2* which had two subtypes represented by four and one isolates. The *emm* sequences of three isolates were novel with reference to CDC and GenBank databases and therefore constitute new *emm* subtypes. These were deposited in the GenBank database with accession numbers JF345261, JF345262 and JF345263 and in the CDC database as new *emm* subtypes. Their new designations and specific changes in amino acid sequence are given in Table 1.

Figure 1 shows the temporal distribution of *emm* types over the study period. Half of the 110 isolates were recovered during 2000–2002 (17, 19, 19 isolates, respectively) and these exhibited the greatest diversity in type distribution. Type *emm1* was found in all but two years, whereas *emm12* and *emm4* emerged later in 2000 and 2001, respectively, and *emm22* disappeared in later years.

Mucoid phenotype, HA content and correlation with infection site and *emm* type

Inspection of colony morphology showed that three isolates (nos. 25, 128, 147) were mucoid in appearance. The HA content of all 110 isolates was quantified and capsulate strains were distinguished from non-capsulate strains by HA values of >10 fg/c.f.u [8, 25]. Mucoid isolate nos. 25 and 128 had an HA content of 11·8 and 23·4 fg/c.f.u, respectively, but isolate no. 147 had only 8·4 fg/c.f.u in spite of its mucoid appearance. The latter isolate was from a sterile site and type *emm1* and the former two were from the pharynx and *emm12*. The HA content for the remainder of the isolates ranged between 0·2 and 8·4 fg/c.f.u with significantly higher values observed

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Table 1. emm-*type distribution in invasive and non-invasive clinical isolates in Chile, 1996–2007*

Type	No. of isolates (%)	No. sterile-site isolates (%)	No. pharyngeal isolates (%)	Subtypes (no. of isolates)	Amino acid substitutions present in new subtypes* (accession no.)
emm12	21 (19·1)	5 (16·7)	16 (20)	12·0 (16)	
				12·57 (1)	Gln[49]Leu (JF345261)
				12·21 (1)	—
				12·56 (1)	His[35]Leu, Asn[46]Ile (JF345262)
				12·5 (2)	—
emm1	16 (14·5)	8 (26·7)	8 (10)	1·0 (13)	—
				1·38 (2)	—
				1·47 (1)	—
emm4	13 (11·8)	0 (0)	13 (16·2)	4·0 (13)	—
emm28	9 (8·2)	3 (10)	6 (7·5)	28·0 (9)	—
emm22	8 (7·3)	3 (10)	5 (6·2)	22·11 (8)	Thr[22]Ile (JF345263)
emm6	7 (6·4)	2 (6·7)	5 (6·2)	6·0 (3)	—
				6·1 (1)	—
				6·4 (3)	—
emm75	6 (5·5)	4 (13·3)	2 (2·5)	75·0 (6)	—
emm2	5 (4·5)	0 (0)	5 (6·2)	2·0(4)	—
				2·2 (1)	—
emm77	5 (4·5)	0 (0)	5 (6·2)	77·0 (5)	—
emm9	4 (3·6)	0 (0)	4 (5)	9·0 (4)	—
emm94	3 (2·7)	0 (0)	3 (3·8)	94·0 (3)	—
emm11	3 (2·7)	3 (10)	0 (0)	11·0 (3)	—
emm78	2 (1·8)	0 (0)	2 (2·5)	78·3 (2)	—
emm87	2 (1·8)	0 (0)	2 (2·5)	87·0 (2)	—
emm3	2 (1·8)	0 (0)	2 (2·5)	3·1 (2)	—
emm48	1 (0·9)	1 (3·3)	0 (0)	48·1 (1)	—
emm73	1 (0·9)	0 (0)	1 (1·2)	73·0 (1)	—
emm81	1 (0·9)	1 (3·3)	0 (0)	81·4 (1)	—
emm89	1 (0·9)	0 (0)	1 (1·2)	89·0 (1)	—
Total	110	30	80		

* Differences with respect to the reference subtype sequence of the CDC database. Amino acid positions correspond to the first 50 amino acids of the mature protein. Gln[49]Leu, glutamine at position 49 changed to leucine; His[35]Leu, histidine at position 35 changed to leucine; Asn[46]Ile, asparagine at position 46 changed to isoleucine; Thr[22]Ile, threonine at position 22 changed to isoleucine.

for sterile-site isolates compared to pharyngeal isolates ($P=0{\cdot}0374$) (Fig. 2*a*). There was no statistical difference between HA content and *emm* type by ANOVA analysis ($P=0{\cdot}9128$) (Fig. 2*b*). Forty-six (41·7%) isolates were resistant to erythromycin and almost half of these were *emm4* and *emm12* (Fig. 2*c*). Erythromycin-resistant isolates had significantly lower HA content than susceptible isolates (Fig. 2*d*).

Sequence analysis of the *csrR* gene

The coding region of the *csrR* gene of all isolates was sequenced to determine if the increased expression of HA of the culturally mucoid strains was due to point mutations, i.e. single nucleotide polymorphisms (SNPs), in this gene. The majority (77/110) of isolates had SNPs in the *csrR* gene; they had combinations of 1–4 SNPs. Each combination was defined as an allelic type (AT) (Table 2). Most SNPs did not result in an amino acid substitution but four of them did produce amino acid substitutions: methionine-170 for isoleucine (Met[170]Ile), valine-173 for alanine (Val[173]Ala), arginine-94 for cysteine (Arg[94]Cys) and glycine-91 for aspartic acid (Gly[91]Asp) (Table 2). Amino acid substitutions Met[170]Ile and Val[173]Ala were located in the DNA-binding domain and found in non-mucoid isolates only whereas Arg[94]Cys and Gly[91]Asp were located in the active site of the

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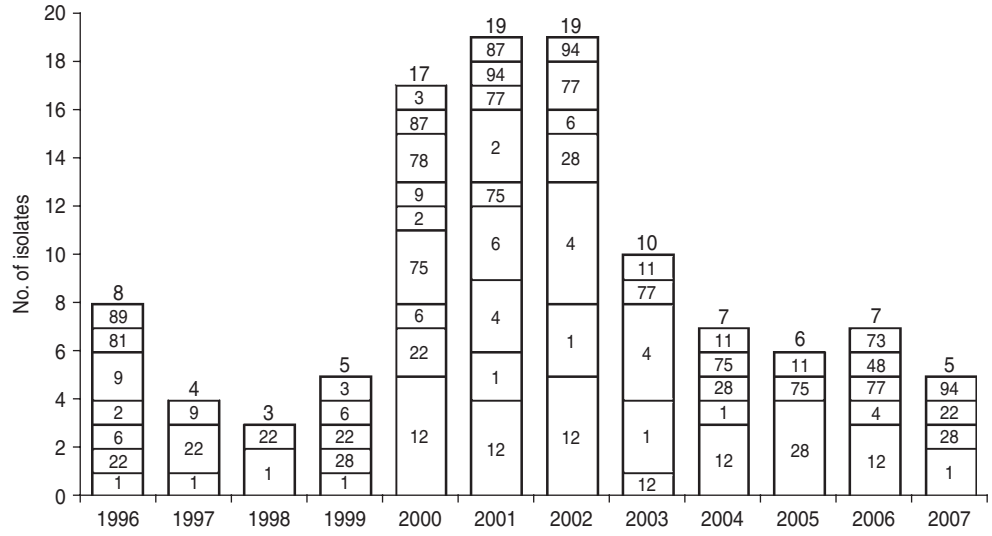

Fig. 1. Temporal distribution of *emm* types in the group A streptococcus population. The number of isolates from each year is shown above each bar and the corresponding *emm* types are shown inside bars.

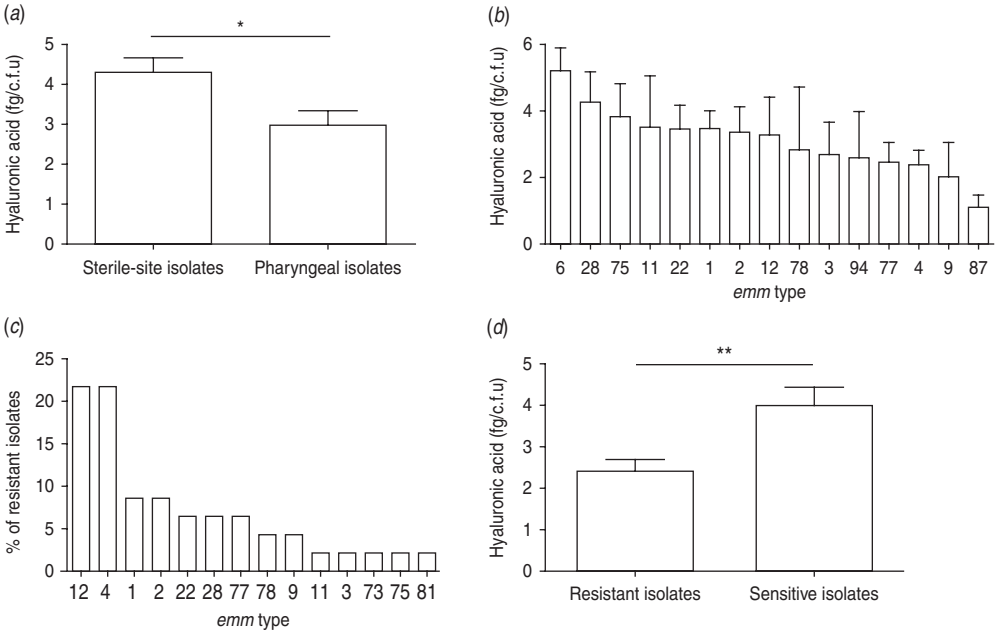

Fig. 2. Hyaluronic acid (HA) content, *emm* type and erythromycin resistance of 110 group A streptococcus clinical isolates. HA content (fg/c.f.u) of (*a*) pharyngeal and sterile-site isolates and (*b*) most prevalent *emm* types. (*c*) *emm*-type distribution among 46 erythromycin-resistant isolates, and (*d*) HA content of erythromycin-resistant and susceptible isolates. Data are means of two or three independent experiments and bars represent the standard error (Student's *t* test: * $P<0{\cdot}05$, ** $P<0{\cdot}005$).

regulator and were present only in mucoid isolates nos. 25 and 128, respectively; no mutation was identified in mucoid isolate no. 147. The novel *csrR* gene sequences were deposited in the GenBank database with accession numbers JF345257, JF345258, JF345259 and JF345260.

Association of *emm* type with *csrR* allele types

A dendrogram was constructed based on the *csrR* gene sequences to analyse the AT distribution of *csrR* gene in the population and its association with *emm* type (Fig. 3). Most ATs were heterogeneous and not

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Table 2. *Allele types of* csrR *gene found in the group A streptococcus population*

Allele type (AT)	Single nucleotide polymorphism (SNP) combination	Amino acid substitution	Colony	Accession no.*
AT-A	T518C	Val[173]Ala	Non-mucoid	JF345259
AT-B	T78G	—	Non-mucoid	—
AT-C	C567T	—	Non-mucoid	—
AT-D	T108C, G222A, T363C, T507C	—	Non-mucoid	—
AT-E	T108C, C396T, T507C, T516C	—	Non-mucoid	—
AT-F	T507C	—	Non-mucoid	—
AT-G	T507C, G510T	Met[170]Ile	Non-mucoid	JF345258
AT-H	T108C, T507C	—	Non-mucoid	—
AT-I	T78G, C280T	Arg[94]Cys	Mucoid	—
AT-J	T78G, G272A	Gly[91]Asp	Mucoid	JF345257
AT-K	G153A	—	Non-mucoid	—
AT-L	T78C	—	Non-mucoid	JF345260
AT-M	T81C	—	Non-mucoid	—
AT-N	T507C, T579C	—	Non-mucoid	—
AT-O	—	—	Non-mucoid†	—

The SNPs producing amino acid substitutions are underlined.
* Accession no. is given for the novel sequences found in this work.
† Isolate no. 147 was the only mucoid strain of the type AT-O.

associated with a particular *emm* type as exemplified by the most frequent AT-O, where 11 different *emm* types were identified, although 36% were of type *emm1*. However, it should be noted that all AT-L and AT-M isolates were type *emm28* and all AT-E isolates were type *emm6*; other associations were 62% of AT-C isolates and 75% of AT-D isolates were of types *emm12* and *emm4*, respectively. In order to statistically ascertain the association between ATs of *csrR* gene and *emm* type, ATs were chosen as the genotypically distinct units for comparison and the *emm* type as the variable unit. Pairwise associations between ATs represented by more than five isolates (AT-O, AT-C, AT-F, AT-D, AT-G) and the more represented *emm* types of these ATs, revealed statistically significant levels of non-random association between AT-O and *emm1*, AT-C and *emm12*, AT-F and *emm22*, AT-D and *emm9*, and AT-G and *emm77* (Table 3). Type *emm12* was significantly associated with AT-C compared to other ATs, *emm4* with AT-D, and *emm77* with AT-G. However, type *emm1* was significantly associated with AT-O compared to AT-C and AT-D and similarly, *emm22* with AT-F compared to AT-C and AT-O (Table 3).

DISCUSSION

This is the first report of *emm* types and HA content of GAS strains in Chile determined in a population of more than 100 strains isolated over more than a decade. There are temporal variations in the *emm*-type distribution which may be due to epidemiological changes of GAS. In our study, nine of the sterile-sites isolates came from blood cultures and four (44%) of them were of type *emm1* in accordance with published reports [5, 26]. The data presented here indicate that *emm*-type distribution in Chile is similar to that reported for Latin America and developed countries [14]. According to data reported by Espinosa *et al.* [27], nine of the ten most frequent *emm* types in Chile and Mexico are common to both countries. However, a similar comparison with India shows only three *emm* types in common [28]. It appears that *emm*-type distribution among invasive isolates is more conserved in different countries as the most frequent types of invasive infections in Argentina [29], USA [30], Mexico [27] and Italy [31] are *emm1* and *emm12*, the same as in Chile. The oligoclonal distribution of *emm* types in Chile is similar to that reported from Belgium [32] and the USA [30] but in contrast with that reported from Brasilia (Brazil) where the most abundant *emm* type had a frequency of only 8·5% which is more indicative of a polyclonal distribution [32] as also found in India and Lebanon [28, 33]. *emm*-type distribution possibly reflects the type of population of a country or region and those regions which have experienced extensive recent migrations increasing host population diversity thus have a greater

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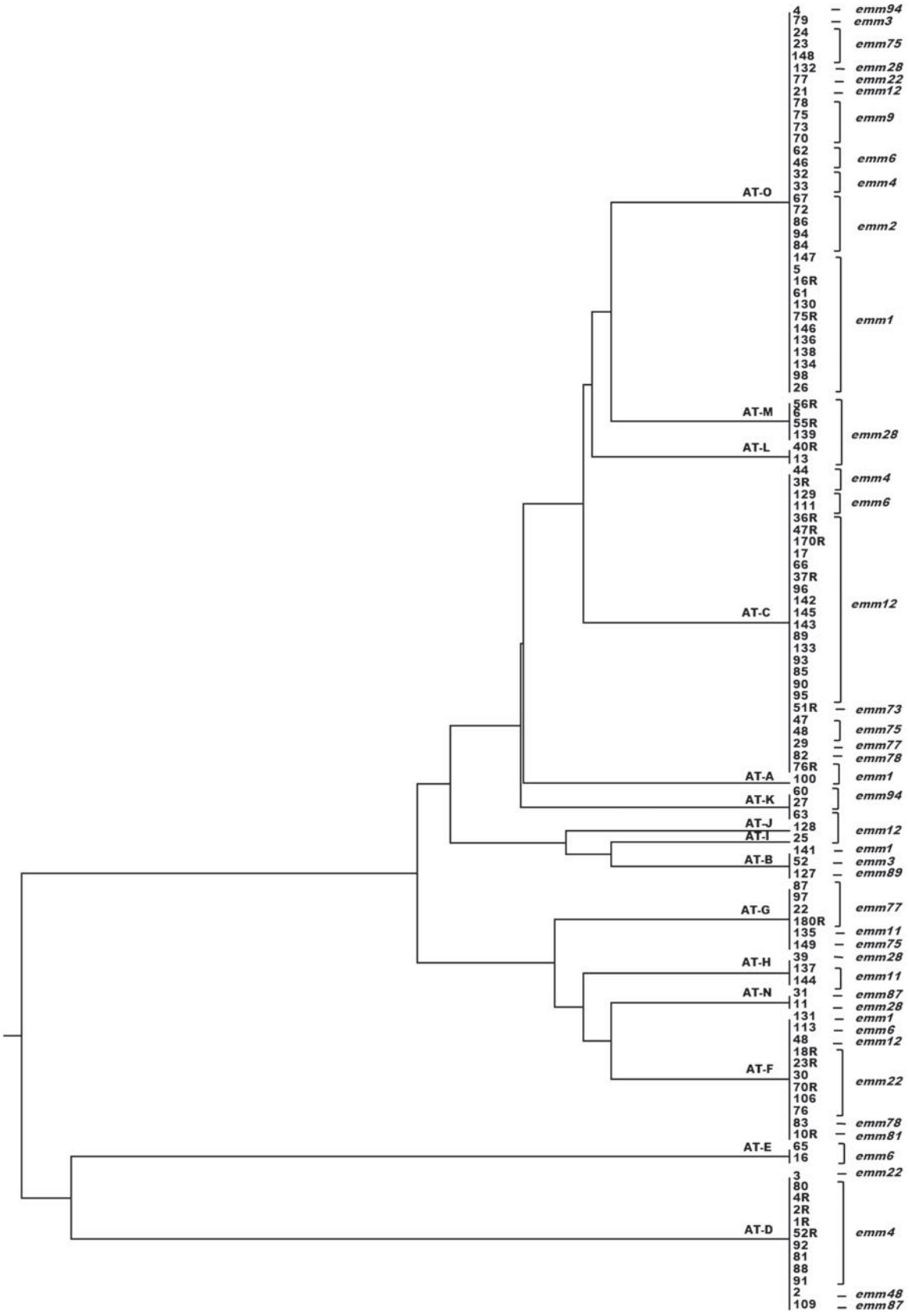

Fig. 3. Dendrogram from UPGMA cluster analysis of the *csrR* gene sequence of 110 group A streptococcus isolates showing allele type (AT) and *emm* type.

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Table 3. *Statistical analysis of allele-type and* emm*-type associations in isolates of distinct allele types*

	P value				
	emm1	*emm12*	*emm22*	*emm4*	*emm77*
O *vs.* C	0·0091	0·0001	n.s.	n.s.	n.s.
O *vs.* F	n.s.	n.s.	0·0004	n.s.	—
O *vs.* D	0·0396	n.s.	n.s.	0·0001	—
O *vs.* G	n.s.	n.s.	n.s.	n.s.	0·0001
C *vs.* F	n.s.	0·0074	0·0004	n.s.	n.s.
C *vs.* D	n.s.	0·0009	n.s.	0·0002	n.s.
C *vs.* G	n.s.	0·0182	—	n.s.	0·0017
F *vs.* D	n.s.	n.s.	n.s.	0·0011	—
F *vs.* G	n.s.	n.s.	n.s.	—	0·0125
D *vs.* G	—	—	n.s.	0·0124	0·0092

χ^2 analysis (two-tailed) with Yates' correction for sample size. $P<0{\cdot}05$ indicates that null hypothesis for random assortment is rejected.
$P>0{\cdot}05$, not significant (n.s.)

diversity of *emm* types. This could also be due to ethnic diversity of geographical regions. The low diversity of *emm* types in Chile may therefore reflect the homogeneous nature and stability of its population.

The average HA content did not vary significantly by *emm* type, possibly because differences were not detectable due to the low number of isolates of some *emm* types. However, we found a significant correlation between increased amount of capsular HA and sterile sites of isolation (invasiveness). This issue has been addressed by Schrager *et al*. [8] who suggested that HA capsule was a virulence factor in soft-tissue infections as capsulate strains avoid uptake by epithelial cells in contrast to unencapsulated ones and this could favour entrance through an extracellular route leading to tissue necrosis and spread-through infection. The analysis of mutations in CsrR repressor showed that mutations Met^{170}Ile and Val^{173}Ala are located in the C-terminal region of the CsrR repressor involved in DNA binding. Isolates bearing these mutations are non-mucoid perhaps because both amino acids are not directly involved in DNA binding [34]. Mutations Arg^{94}Cys and Gly^{91}Asp are located in the active site of the regulator and both of these are associated with increased amount of HA and mucoid colony phenotype. It has been reported that mutations in this region of the protein confer mucoid character [11]. However, mucoid isolate no. 147 described here has no mutations in its *csrR* gene which suggests that factors other than repressor inactivation might contribute to increased capsule synthesis.

M protein is a target of the protective immune response, and amino acid substitutions in this protein would allow the pathogen to evade the immune response. The *emm* gene is therefore subjected to a high selective pressure and may change rapidly resulting in *emm*-type variation among isolates. By contrast, housekeeping genes are not subject to the same pressure and change at a much lower rate. The *csrR* gene, although not a housekeeping gene, encodes a global regulator which is not expected to be under high selective pressure as the *emm* gene. Therefore, the association between *emm* type and *csrR* sequence is a valid means of estimating horizontal gene transfer in the GAS population. Genetic linkage between *emm* gene markers and *spe* alleles have been described in GAS isolates [35] but to our knowledge this is the first report of a correlation existing between *emm* and *csrR* genes. The non-random association of *csrR* alleles with *emm* types could be due to the strong clonality of GAS that causes *csrR* and *emm* genes to change together. These observations support the concept that free horizontal gene transfer is hindered by certain barriers, otherwise many random combinations of ATs and *emm* types would be observed. This picture is compatible with an oligoclonal GAS population, as observed in Chile, in which there is a lack of incoming new strains due to its geographical and social characteristics. The geographical isolation of Chile is supported by the absence of both community-acquired methicillin-resistant *Staphylococcus aureus* and carbapenemase-producing Enterobacteriaceae which are very frequent in other South American countries including Argentina [36, 37], Uruguay [38], Colombia [39, 40] and Brazil [41, 42].

The most recent published study of *emm*-type distribution in Chile in 2001 [43], addressed antimicrobial-resistant isolates only. Our data suggest that the *emm*-type distribution among erythromycin-resistant isolates has changed since then as type *emm2*, which represented 73% of resistant isolates in the period 1994–1998 has decreased to 7% in the present study. This could be due to the decrease of erythromycin resistance observed in recent years [44].

There is no GAS vaccine yet available although these organisms cause more deaths than, e.g. *Neisseria meningitidis*, against which a vaccine is available. A multivalent vaccine based on purified M-protein peptides of the 26 most prevalent *emm* types is currently undergoing the Phase II stage of development in the USA [45]. If the US vaccine was used in Chile, it would protect against only 58% of the most frequent

emm types found in this study. It is necessary to know the *emm* types that are present in the population in order to allow the evaluation of the effectiveness of future GAS vaccines in this population. Continued surveillance of *emm*-type distribution among invasive and non-invasive GAS is therefore required to guide the design and use of potential vaccines.

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DECLARATION OF INTEREST

None.

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