International Quality Assurance Study for Characterization of *Streptococcus pyogenes*[▽]

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Surveillance of group A streptococcal (GAS) infections was undertaken as a major component of the European Commission-funded project on severe GAS disease in Europe (strep-EURO). One aim of strep-EURO was to improve the quality of GAS characterization by standardization of methods. An external quality assurance study (EQA) was therefore carried out to evaluate current global performance. Eleven strep-EURO and seven other streptococcal reference centers received a panel of 20 coded GAS isolates for typing. Conventional phenotypic typing (based on cell surface T and M protein antigens and opacity factor [OF] production) and molecular methods (*emm* gene typing) were used either as single or combined approaches to GAS typing. T typing was performed by 16 centers; 12 centers found one or more of the 20 strains nontypeable (typeability, 89%), and 11 centers reported at least one incorrect result (concordance, 93%). The 10 centers that tested for OF production achieved 96% concordance. Limited availability of antisera resulted in poor typeability values from the four centers that performed phenotypic M typing (41%), three of which also performed anti-OF typing (typeability, 63%); however, concordance was high for both M (100%) and anti-OF (94%) typing. In contrast, the 15 centers that performed *emm* gene sequencing achieved excellent typeability (97%) and concordance (98%), although comparison of the performance between centers yielded typeability rates from 65 to 100% and concordance values from 83 to 100%. With the rapid expansion and use of molecular genotypic methods to characterize GAS, continuation of EQA is essential in order to achieve international standardization and comparison of type distributions.

Group A streptococci (GAS; *Streptococcus pyogenes*) cause a wide range of infections globally, from acute pharyngitis and impetigo to more severe diseases such as rheumatic fever, bacteremia, and toxic shock syndrome (3). It is therefore essential to monitor trends by typing strains, an approach that was initially pioneered by Rebecca Lancefield in 1920 (12). Classical characterization of GAS uses phenotypic techniques that are mainly based upon the cell surface T and M protein antigens (9). In GAS, type-specific T-protein antigens are basic markers for typing and can be divided into approximately 30

different T types. Typing of the T proteins uses polyvalent pooled and monovalent antisera in a slide agglutination test (9). The M protein is a major virulence factor of GAS and, due to its hypervariable amino terminus, there are approximately 90 validated and specific M types (7). M type-specific antisera are used in a precipitin reaction against GAS acid extracts (12).

The serum opacity factor (OF), a type-specific cell surface enzyme, is produced by approximately half of the designated M types; the enzyme causes mammalian sera to become opaque (2). Thus, a GAS strain can either be OF positive or negative, and this correlates with the M type (9). Furthermore, the OF exhibits type-specific markers that can be used serologically in the anti-OF inhibition test. However, M and anti-OF antisera are not commercially available and are very labor-intensive and costly to produce and maintain.

In an effort to make GAS characterization more accessible to other laboratories, molecular methods targeting the hypervariable N terminus area of the gene for the M protein (*emm*) were developed (1, 16, 22). Determination of the *emm* gene sequence has become a widely used alternative to M typing for GAS characterization, and there are currently more than 110 officially designated *emm* types (*emm*-1 through *emm*-124) (10). The *emm* types are further divided into subtypes that are explicitly based on minor sequence variation within the type-

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specific hypervariable region of the gene, and there are currently over 800 *emm* sequence subtypes that have been described. There is a dedicated, curated website maintained by the Centers for Disease Control and Prevention (CDC) with a searchable database of all validated M and *emm* types, as well as provisional types and subtypes yet to be officially designated (<http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm> [last accessed 27 February 2007]).

When sequencing of the *emm* gene was first introduced, it was expensive, technically demanding and lengthy; therefore, *emm* screening assays were developed in the mid 1990s. These included *emm* reverse line blot hybridization and *emm* PCR enzyme-linked immunosorbent assay (ELISA); both methods involve the PCR amplification of the *emm* gene and hybridization to *emm* type-specific oligonucleotide probes (11, 18).

Combinations of the above methods are currently used worldwide to characterize GAS, and it is therefore essential for epidemiological and microbiological surveillance to evaluate and harmonize typing methods so as to allow a meaningful comparison of results between typing centers. In addition, with continuing interest in the development of effective GAS vaccines based on M proteins, it is paramount to monitor M- and *emm*-type distributions to track emerging, less-predominant types that may impact on public health and vaccine formulation (15). Documentation and monitoring of GAS typing by external quality assurance (EQA) programs have been performed previously. Five GAS EQA panels were distributed among six centers worldwide between 1997 and 1999, at a time when *emm* gene typing was being introduced within international reference centers (5). It was concluded that, for optimal surveillance, EQA assessment of GAS characterization should be maintained regularly among typing centers despite the cumbersome and costly procedure for the distribution of isolates (5).

A European Commission Fifth Framework program (QLK2.CT.2002.01398) for severe GAS disease was launched on 1 September 2002 (strep-EURO) (19). The major objective of this 4-year project, which involved 11 countries and 12 centers, was to enhance understanding of the epidemiology of GAS invasive disease in Europe. One of the specific targets of strep-EURO was to improve GAS strain characterization by harmonizing methods and an EQA. Other international streptococcal reference centers were invited to participate. The overall aim was to evaluate the laboratories' capabilities and current methodologies for GAS characterization on an international level.

MATERIALS AND METHODS

Participants. The participating centers included 11 strep-EURO centers in Lund, Sweden; Aachen, Germany; London, United Kingdom; Prague, Czech Republic; Athens, Greece; Rome, Italy; Helsinki, Finland; Copenhagen, Denmark; Bucharest, Romania; Stockholm, Sweden; and Paris, France. The remaining center from Nicosia, Cyprus, was unable to participate, since a fully working streptococcal reference center was not established at the time of the EQA. A further seven streptococcal reference centers were invited to participate; these centers were located in Minneapolis, MN; Atlanta, GA; Porirua, New Zealand; Edmonton, Alberta, Canada; Bilthoven, The Netherlands; Jerusalem, Israel; and Antwerp, Belgium. The EQA was established and coordinated by the Streptococcus and Diphtheria Reference Unit in London, United Kingdom, which coordinated the 1997-1999 EQA study (5).

Bacterial strains and transport conditions. A total of 20 coded strains of *S. pyogenes* were sent to all centers for typing (Table 1). Sixteen were selected from

TABLE 1. Intended results of the 20 EQA GAS strains (from London, United Kingdom)

EQA strain	T type	OF testing ^a	M type as determined by immunodiffusion	Anti-OF type	<i>emm</i> type
EQA-SA1	3/13/B3264	N	NT ^c		43
EQA-SA2	28	P	NT	NT	96
EQA-SA3	Not tested ^b	N	18		18
EQA-SA4	B3264	P	NT	NT	102
EQA-SA5	13	N	83		83
EQA-SA6	1	N	1		1
EQA-SA7	3/13/B3264	N	NT		101
EQA-SA8	28	P	NT	87	87
EQA-SA9	2	P	2	2	2
EQA-SA10	12	N	12		12
EQA-SA11	28	P	R28	28	28
EQA-SA12	3/13/B3264	P	NT	89	89
EQA-SA13	5	P	NT	82	82
EQA-SA14	5	N	5		5
EQA-SA15	11	P	NT	78	78
EQA-SA16	B3264	N	NT		101
EQA-SA17	6	P	NT	NT	109
EQA-SA18	5/27/44	N	NT		116
EQA-SA19	3	N	NT		122
EQA-SA20	1	N	NT		105

^a N, negative reaction; P, positive reaction.

^b EQA-SA3 was not tested with T18 antisera before being sent to participants.

^c NT, strain nontypeable by this method.

clinical isolates submitted from hospital laboratories in England and Wales to the Streptococcus and Diphtheria Reference Unit, Health Protection Agency, during 2003 and comprised M and *emm* types 1, 2, 5, 12, 18, 28, 43, 78, 82, 83, 87, 89, 96, 101 (two strains), and 102. Four were reference strains of the recently designated *emm* types 105, 109, 116, and 122 (8). The GAS strains chosen did not represent any particular regional M or *emm* type distribution and were selected for their T and M profile diversity. Isolates were grown and transported on Columbia blood agar slabs (Media Services, Health Protection Agency Centre for Infections, London, United Kingdom) to the 18 centers in 16 countries.

Questionnaire assessments. A questionnaire was sent to all participants 12 months before dispatch of the EQA panel for completion and return to the coordinating center. The information requested concerned each center's current laboratory diagnostic and typing methodologies for GAS, the reference services provided, and the numbers of GAS isolates examined in each center from 1999 to 2003. Aspects of the EQA exercise that were assessed included all typing results generated; details of methods, media, typing sera, and control strains used; the interpretation of data; and problems encountered.

Analysis. The typeability and concordance was calculated for each method used and for those centers using the *emm* gene sequencing method (21). The typeability formula is: typeability = N_t/N , where N_t is the number of isolates assigned a type, and N is the number of isolates tested. Typeability values are shown as percentages for the present study, and values approaching 100% represent the optimal usefulness of a method. The concordance values were calculated by using the formula concordance = N_c/N (also shown as a percentage), where N_c is the number of isolates assigned the same type as the intended result, and N is the number of isolates assigned a type. For reliable definitive typing, concordance should ideally be greater than 95% (21).

RESULTS

The status and capabilities of the 18 participating centers were initially assessed. Sixteen centers are National Reference Centers for streptococci; five of these are also designated World Health Organization (WHO) Collaborating Centers. The duration of experience and/or designation as a nationally recognized reference center for characterizing GAS isolates varied widely from 2 to 60 years. All 18 centers performed phenotypic tests at various levels (T typing, OF typing, and/or

TABLE 2. GAS typing methods performed in 18 reference centers

Typing methods	No. of centers	% Typeability (no. of positive results/total no. of tests performed) ^a	% Concordance (no. of matched results/total no. of results)
T typing ^b	16	89 (283/319)	93 (263/283)
OF testing	10	100 (180/180)	96 (172/180)
Serum OF (<i>sof</i>) gene detection and sequencing ^b	1	100 (19/19)	95 (18/19)
M type immunodiffusion	4	41 (21/51)	100 (21/21)
Anti-OF typing ^c	3	63 (17/27)	94 (16/17)
<i>emm</i> PCR ELISA ^d	1	42 (5/12)	100 (5/5)
<i>emm</i> reverse line blot hybridization ^e	2	50 (20/40)	70 (14/20)
<i>emm</i> gene sequencing ^{b,f}	15	97 (275/284)	98 (268/275)

^a The number of results do not include any nontypeable or auto-agglutinating isolates.

^b One strain was nonviable in one center.

^c Anti-OF typing can only be performed on OF-positive isolates ($n = 9/20$ from the panel).

^d This method was used as a screening step after conventional typing methods are used, and nontypeable strains are then sequenced.

^e One country used this method as a screening step before the *emm* sequence is determined on nontypeable strains.

^f Some centers used other tests to determine the M/*emm* type initially; therefore, not all 20 isolates underwent *emm* sequencing.

M typing), and 16 centers applied genotypic methods to determine the *emm* gene type. The annual average number of GAS isolates submitted to each center varied considerably from 24 (Greece) to approximately 1,600 (United States) during the period from 1998 to 2002. These differences in numbers were largely due to the population coverage for each country and surveillance mechanisms in place during that period. The results of the EQA exercise from the 18 centers were received at the coordinating center in the United Kingdom for analysis and comparison.

T typing. Sixteen centers determined T types by a slide agglutination method; thirteen centers used commercially available specific antisera (SevaPharma, Prague, Czech Republic, or Denka Seiken, Tokyo, Japan). Three centers used antisera produced in-house. Twelve centers found one or more of the strains nontypeable by T typing, which resulted in an overall typeability value of 89% (Table 2). Discrepant results were reported from 11 centers, producing a concordance value of 93%. However, results from the individual centers that were concordant with the intended result ranged from 80 to 100%.

OF reaction and *sof* sequencing. Ten of eighteen centers performed phenotypic OF testing, and one center (Atlanta, GA) genotypically tests for the presence of *sof*, the gene encoding the OF enzyme; where present, the gene was sequenced (Table 2). All 11 centers recorded an OF or *sof* result for the 20 strains, although 3 centers reported discrepant results (typeability, 100%; concordance, 96%).

M-type precipitin and anti-OF typing. Only four centers performed classical M typing, and three also undertook anti-OF typing (Table 2). Overall, the typeability values for M and anti-OF typing were low (41 and 63%, respectively) due to the limited availability of M and anti-OF antisera in each center. In addition, these centers did not determine the M and/or anti-OF type for all 20 strains; 8 of 20 strains could not

be typed using specific M or anti-OF antisera since they belonged to the higher *emm* types; these types have yet to be validated as official M types (7). Although M and anti-OF typing gave low typeability values, the tests achieved high concordance values of 100% for M typing and 94% for anti-OF typing.

***emm* PCR ELISA and *emm* reverse line blot hybridization.** The *emm* PCR ELISA was used in only one center (Table 2; London, United Kingdom) for the examination of strains that were negative with the classic serotyping methods (M type immunodiffusion and anti-OF typing). ELISA-negative isolates were subsequently sequenced to elucidate their *emm* gene type. The ELISA produced a low typeability value (42%), but as for M typing, all of the positive results matched with the intended result (concordance of 100%). Two centers used *emm* reverse line blot hybridization, and one of those centers also used sequencing to resolve nontypeable strains or those presenting with cross-reactions to more than one probe (Table 2). This method also gave a low typeability value (50%) where one of the centers reported incorrect results for several strains, thus generating a low concordance value of 70%.

***emm* gene sequencing.** Fifteen of eighteen centers determined the *emm* sequence for the EQA panel by direct sequencing (Table 2). Both the typeability and the concordance were high at 97 and 98%, respectively, but four centers reported an incorrect *emm* type for one or more strains: *emm* 68 for EQA-SA2 (*emm* 96, OF⁺, T28); *emm* 96 for EQA-SA5 (*emm* 83, OF⁻, T13); *emm* 89, 109, or 116 for EQA-SA16 (*emm* 101, OF⁻, TB3264); and *emm* 116/101 and 22 for EQA-SA17 (*emm* 109, OF⁺, T6). There was also variation in the interpretation of *emm* subtypes (e.g., for EQA-SA14, *emm* 5, 5.5, 5.27, 5.32, and 5.37 were reported), which highlighted the difficulties when assigning subtypes. Fourteen of fifteen centers used the CDC *Streptococcus pyogenes emm* sequence database to assign *emm* types, and a few centers also used the GenBank BLAST facility; one center used only the latter.

Performance between centers. To measure the overall performance of each center, *emm* gene sequencing was chosen, since 15 of the 18 centers performed this test (Table 3). Centers not assessed included one that typed to the T-type level, one that used classical M serotyping only, and one that used *emm* reverse line blot hybridization. The typeability from the 15 centers varied from 65 to 100%, although 13 of the 15 centers achieved 100% and reported results for all of the strains tested. The concordance value also varied from 83 to 100%, although 11 of 15 centers gave results concordant to those intended and achieved 100%.

DISCUSSION

The last GAS EQA program from 1997 to 1999 provided an opportunity to evaluate the feasibility of *emm* gene sequencing as an alternative to M typing among key international streptococcus reference laboratories and resulted in the publication of a standardized process for assigning *emm* types (5). Since then, many reference and research laboratories have now embraced *emm* gene sequencing, and various studies have been published (2, 4, 6, 14, 17). The strep-EURO project has strengthened links between centers and has established a network of European streptococcal reference centers. Thus, it was

TABLE 3. Performance of *emm* gene sequencing in 15 participating centers

Center ^a	% (no. of positive or matched samples/total no. of samples tested)	
	Typeability	Concordance
P1*	100 (7/7)	100 (7/7)
P2	100 (20/20)	100 (20/20)
P3†	100 (19/19)	100 (19/19)
P5	100 (20/20)	100 (20/20)
P6	100 (20/20)	100 (20/20)
P7	100 (20/20)	100 (20/20)
P8	100 (20/20)	100 (20/20)
P9	100 (20/20)	100 (20/20)
P10	100 (20/20)	95 (19/20)
P11	65 (13/20)	100 (13/13)
P12	100 (20/20)	95 (19/20)
P13	100 (20/20)	90 (18/20)
P14	90 (18/20)	83 (15/18)
P16	100 (20/20)	100 (20/20)
P17*	100 (18/18)	100 (18/18)
Overall	97 (275/284)	98 (268/275)

^a *, These centers used other tests to determine the M and *emm* type initially; therefore, not all 20 isolates underwent *emm* sequencing. †, One strain was nonviable at this center.

both timely and appropriate for a further EQA exercise to be undertaken so that the capabilities of centers and the assessment of GAS typing methods could be evaluated. The capabilities of the 18 centers assessed varied in terms of the phenotypic and/or genotypic methods performed. This was correlated to the differences in workloads and, as an example, the WHO Collaborating Centers perform most of the methods listed in Table 2 and could examine up to 1,600 GAS isolates a year. T typing and *emm* gene sequencing were performed in the majority of centers, and many centers wanted to expand their GAS characterization repertoire.

The phenotypic methods—T, M, and anti-OF typing—are reliant upon viable GAS organisms to express the respective proteins and good-quality, well-characterized antisera. In the EQA exercise, there was 100% typeability for OF determination, since the test, in contrast to the other three methods, did not require complex reagents such as type-specific sera. The T type results from the present study were mostly concordant, although some cross-reactions occurred. This highlighted the need for careful interpretation by experienced staff if T typing is used as the sole strain characterization method in epidemiology studies. Three centers reported T18 for EQA-SA3 (M/*emm* 18, OF⁻, T nontypeable/18), whereas the majority of centers did not include T18 antisera in their T typing scheme. The mucoid nature of M/*emm* 18 strains makes them difficult to type; therefore, if a good quality T18 antiserum has recently become available, centers should include this in their T antiserum repertoire. A few centers who reported problems are currently reviewing their T typing methodology to attain higher qualitative results. In addition, one manufacturer has noticed an over-reactivity of their T8 antisera and is working to correct this (E. Kaplan, unpublished data.). The overall typeability of the M and anti-OF tests was comparatively lower than in the previous GAS EQA assessment (5) (50% versus 72%), which was due to depleting stocks of in-house produced sera and the

inclusion of new *emm* types in the EQA panel. Only four centers (three for anti-OF testing) performed these tests, which emphasized the decline in the use of classic methods. However, phenotypic methods are essential for the validation of new M types and were used to confirm 22 additional M types to the Lancefield classification scheme (8). The depletion of serum stocks will be problematic for future validation of new M types.

The *emm* PCR ELISA and *emm* reverse line blot hybridization assays generated poor typeability values in the EQA exercise, since the probes in routine use were designed for the major *emm* types seen in the respective countries. These hybridization methods, therefore, are ideally used for screening, and nontypeable strains should be elucidated further by *emm* gene sequencing. If this approach is not undertaken, then this may compromise surveillance in some countries, since travel and immigration could potentially introduce uncommon types into the circulating GAS population.

Emm gene sequencing was used in most centers, providing high typeability and concordance, as reported in this and the previous GAS EQA exercise (5). However, some participants either reported the *emm* type (e.g., *emm* 1 or *emm* 83) or the *emm* subtype (e.g., *emm* 1.1 or *emm* 83.1), depending upon their interpretation criteria. At the time of the EQA, the curator of the CDC *Streptococcus pyogenes emm* sequence database revised and simplified the designation of *emm* types and, consequently, there was an overlap of two systems to assign both *emm* types and subtypes. This was not initially made apparent to the scientific community and may have been a causal factor in several different *emm* subtypes being reported for the same strain. In addition, most of the discrepant *emm* types reported from four centers were probably due to high similarity matches in the conserved region of the *emm* gene, which is generally downstream of the defined *emm* type.

The precise definition of *emm* types and subtypes requires revision due to the increasing GAS typing community who refer to the expanding *emm* reference database. As a consequence, consultation and frequent communication between centers has been initiated for agreement on a standard criterion for the validation and assignment of *emm* types and subtypes (A. Efstratiou, unpublished data.). The CDC database is more accurate than the GenBank BLAST facility because all *emm* sequences to *emm* 124 have been validated in accordance with the validation procedures and nomenclature of new *emm* sequence types (7). To date, GenBank *emm* sequence data are not updated by a dedicated curator and are not validated; consequently, it is recommended that GenBank should not be used to assign *emm* types (8).

The *emm* gene sequencing method has been shown to be an accurate and reliable alternative for M typing. The production of M sera is laborious, expensive, and only available to a few centers, and this has most likely contributed to an increase in nontypeable isolates that require *emm* gene sequencing (13, 20; A. Efstratiou, unpublished data.). Therefore, although currently costly, *emm* gene sequencing should be viewed as the gold molecular standard for typing GAS, as confirmed in the present study by the excellent typeability and concordance values. However, the importance of the biological significance of M protein and the role of M typing and M antibodies in the study of GAS should not be overlooked.

This GAS EQA exercise, due to the establishment of a network of strep-EURO centers and close communication with WHO Collaborating Centers and other international reference centers, is the largest to date. It encouraged several European centers to introduce *emm* sequencing and other techniques and to improve their methodologies in a constructive and supportive manner. Newly established streptococcal typing facilities can rely on T typing and the OF determination test to form the basis for further characterization.

With the rapid expansion and use of molecular methods to characterize GAS, EQA exercises are essential in order to achieve standardization and direct comparison of type distributions between countries. The implementation of future large-scale EQA exercises has major cost implications. The resurrection of the WHO Ad Hoc Laboratory Working Group on Streptococci from a decade ago may ease this difficult burden, thus furthering progress in streptococcal epidemiology, vaccinology, and laboratory diagnostics.

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