HARMONY – The International Union of Microbiology Societies' European Staphylococcal Typing Network

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Introduction

The HARMONY typing network was part of the European Union (EU) Directorate General XII (now the Directorate-General for Research) funded project "Harmonisation of Antibiotic Resistance measurement, Methods of typing Organisms and ways of using these and other tools to increase the effectiveness of Nosocomial Infection control", awarded in 1999. Other aspects of the project comprised the exploration of the feasibility of developing a consensual approach to infection control guidelines, examining the issues of antimicrobial susceptibility standardisation and developing a tool to facilitate the establishment of effective antibiotic stewardship [1,2].

Many of the typing group participants were also members of the International Union of Microbiology Societies' (IUMS) Staphylococcal Sub-Committee. This was established in the 1970s to ensure that phage typing was standardised globally and to provide propagating phages for phage-typing [3]. Over time phage-typing had become less useful for some strains of methicillin-resistant and, indeed, methicillin-sensitive, *Staphylococcus aureus* (MRSA and MSSA), as they had become non phage-typable [3]. The IUMS Staphylococcal Sub-Committee now included reference laboratories and centres of staphylococcal research excellence with interests in typing staphylococci by molecular techniques which were more effective than phage for typing some staphylococci. When we started the HARMONY project, it was at a time of tremendous advances in molecular typing methods and we thus added new techniques to the HARMONY assessment process as these became relevant and practical propositions. There were also other aims such as, for example, agreeing criteria for referral of isolates to a typing laboratory and an approach to the nomenclature of MRSA strains.

Criteria for referral of isolates to a typing laboratory

When the project started, only two centres had such criteria. These were important in ensuring that typing was being used optimally to investigate suspected outbreaks or emerging new virulent strains or strains resistant to new or multiple antimicrobials. It would also enable comparison of workloads in centres within and between countries. There was thus much interest in developing a consensus regarding such criteria. One of the centres (England) had been particularly successful in reducing MRSA referrals from ca. 48,000/year to ca.12,000/year between 1995 and 2000, and these were therefore the criteria that the group considered [4]. Table 2 shows the final set of criteria that were agreed upon. There were certain caveats to this. Firstly, they were developed before the emergence of community-acquired MRSA (CA-MRSA) in some EU countries and would therefore need to be adapted to ensure that customers were aware of the characteristics of Panton-Valentine leukocidin (PVL) related MSSA and MRSA syndromes [5]. The English laboratory has used separate information forms for toxin-related disease for many years and these have been modified to take into account PVL-positive strains since the project was completed.

Secondly, some countries with a non-endemic MRSA situation requested referral of all individual patient isolates of MRSA to their centre (even those just colonising patients or staff). One centre requested all bacteraemia *S. aureus* isolates be sent to it where results were used for national surveillance purposes. Several centres, of course, also received referrals from their European Antimicrobial Resistance Surveillance participating hospitals and one of these centres also typed these [6]. The existence of such criteria does not mean that they are being implemented correctly and the group emphasised the importance of reviewing and perhaps auditing these criteria regularly. For example, when the criteria were audited in 1998 in England and Wales [4], although the infection control team usually wrote the referral policy and reviewed the results, there were many variations, and often junior or non infection control personnel were involved in making the decisions on referring isolates. If a member of the infection control team was involved, the laboratory was statistically significantly more able to describe the numbers of isolates sent and to reduce these. Those sending less than 150 isolates in a year were also significantly more accurate in estimating what had been sent and less likely to send unnecessary multiple isolates.

Harmonisation of MRSA typing

Initially, all the HARMONY participating centres were using pulsed-field gel electrophoresis (PFGE) to type MSSA and MRSA. The network collected together, for the first time in the EU, important or epidemic MRSA strains. In-house protocols from 10 laboratories in eight European countries were compared by each centre with an agreed "gold standard" PFGE protocol in which many of the parameters had been standardised [7]. Isolates were later added from other countries (Ireland, Scotland, Slovenia, Poland and Portugal).

Table 1

HARMONY International Union of Microbiology Societies (IUMS) typing laboratory network participants

Participants	Organisation	Country
G. Coombs (Resistotyping lead)	Royal Perth Hospital, Perth	Australia
M. Struelens, A. Deplano R. de Ryck	Hôpital Erasme - Centre for Molecular Diagnostic (CMD), Brussels	Belgium
R. Skov, V. Fussing (to 2002)	Statens Serum Institut, Copenhagen	Denmark
B. Cookson (Co-Ordinator), A. Lynch, S. Murchan, P. Kaufmann	Laboratory of Healthcare Associated Infection, Health Protection Agency, London	England
S. Salmenlinna, J. Vuopio-Varkila (Ribotyping lead)	National Public Health Institute, Department of Bacteriology, Helsinki	Finland
N. El Solh (deceased)	Institute Pasteur, Paris	France
W. Witte, C. Cuny	Robert Koch Institute, Wernigerode Branch, Wernigerode	Germany
P.T. Tassios, N.J. Legakis	National and Kapodistrian University of Athens, Athens	Greece
A. Rossney , B. O'Connell	National MRSA Reference Laboratory, St James's Hospital, Dublin	Ireland
W. Hryniewicz	National Medicines Institute, Warsaw	Poland
D. Morrison	Microbiology Department, Stobhill Hospital, Glasgow	Scotland
M. Mueller-Premru	University of Ljubljana, Medical Faculty, Ljubljana	Slovenia
J. Garaizar	Dept. Immunol., Microbiol. y Parasitol., F. Farmacia, UPV/EHU, Vitoria-Gasteiz	Spain
A. Vindel	Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid	Spain
S. Hæggman, B. Olsson-Liljequist	Swedish Institute for Infectious Disease Control, Solna,	Sweden
A. van Belkum W. van Leeuwen (Binary typing lead)	Erasmus MC, Center, Rotterdam	The Netherlands

Table 2

Criteria for referral of isolates to a typing laboratory

Introductory statement To enable us to maintain our current low turn round times and improve the quality of the service, please:
1) Request typing only if you intend to act upon the results.
2) Ensure that the Consultant Microbiologist and/or Infection Control Team have confirmed there are good reasons for submission.
3) For all requests, state hypothesis to be tested, i.e. how typing will make a difference.
4) If in any doubt contact us and ask.
5) In outbreaks ("a temporal and spatial cluster above the normal baseline") please send the minimum number of isolates needed to inform local practice (this should rarely be more than half), and store temporally related isolates.
6) Give priority to isolates that cause invasive or serious infection during the course of an outbreak, but avoid sending multiple isolates from **single patients** or environmental isolates, without discussion with us.
7) Wherever possible, use surrogate markers such as biochemical tests e.g. urease and antimicrobial resistances and include representative isolates with significantly different phenotypes e.g. in antibiotic susceptibilities, pigmentation and/or haemolysis.
8) In endemic situations ("where a hospital is constantly challenged with MRSA in patient re-admissions and inter-hospital transfers"), if surrogate markers are being used to identify any locally endemic strains we are willing to check a few representative isolates for you from time to time, e.g. five isolates every six months.
9) Toxic shock and endocarditis. We would like to receive an isolate from every case of suspected staphylococcal toxic shock and endocarditis for toxin-testing and MIC testing respectively.
10) Anomalous isolates.Please state the anomaly/resistance to be investigated eg slide coagulase negative MRSA, and please check for mixed culture, coagulase, catalase and Gram stain before sending.
11) Antibiotic resistance. Request antibiotic susceptibility tests only when necessary to assist your local studies, e.g. anomalous or doubtful test results, unusual or clinically significant results, necessary quantitation (e.g. MIC of first encountered mupirocin-resistant isolates), unexpected resistance e.g. to vancomycin).

From these discussions, and by testing different reagents and protocols in the central laboratory and then in other centres, it was found that it was not important to standardise some elements of the protocol, such as the type of agarose, DNA block preparation and plug digestion. Other elements were shown to be more critical; namely, a standard gel volume and concentration of agarose, the DNA concentration in the plug, the ionic strength and volume of electrophoresis buffer used the temperature, voltage and switching (pulsing) times during electrophoresis [7]. This "harmonised" approach proved to be extremely successful in establishing agreement, in that members were reluctant to abandon methods that they had developed over many years without good reason.

Exchanges of scientists between laboratories enabled the identification of some of these important variables (e.g. where the temperature of the buffer was monitored). The new "harmonised" protocol was agreed, and further modified in a pilot study between two laboratories (Brussels, Belgium and London, England), which resulted in a good compromise between electrophoresis times and strain discrimination [7]. Again, this was made possible by the funded exchange of workers between these two laboratories. Seven laboratories' gels were found to be of sufficiently good quality to allow comparison of the strains using a computer software program, while two out of twenty gels could not be analysed because of inadequate destaining and DNA overloading. These issues were to a certain extent due to the employment of less experienced student workers, which made the group aware of the importance of a more accreditation-oriented approach. Good quality gels and inclusion of an internal quality control strain (NCTC 8325) were found to be essential before attempting inter-centre PFGE comparisons. We were finally able to track a number of clonally-related strains in multiple countries throughout Europe [7,8] summarised in Table 3. This highlighted the need for closer international collaboration to monitor the spread of current epidemic strains as well as the emergence of new ones.

We also characterised these MRSA strains with a number of other techniques e.g. antimicrobial susceptibility, phenotyping, resistotyping, ribotyping, binary typing [9] and toxin gene detection [7]. We then collaborated with Mark Enright from Imperial College, London, United Kingdom (UK) to analyse a representative sample of MRSA from 11 European countries to compare our standardised PFGE typing to two other typing methods: sequencing of the variable repeat region in the protein A-encoding *spa* gene, and multilocus sequence typing (MLST) combined with PCR analysis of the staphylococcal chromosomal cassette containing the *mec* gene (SCC*mec*) [8]. A high level of discrimination was achieved using each of the three methodologies, with discriminatory indices ranging between 89.5% and 91.9%, with overlapping 95% confidence intervals.

There was also a high level of concordance of groupings made using each method. MLST/SCC*mec* typing distinguished 10 groups, each containing at least two isolates. Interestingly, these corresponded to the majority of nosocomial MRSA clones described in the literature. PFGE and *spa*-typing resolved 34 and 31 subtypes, respectively, within these ten MRSA clones. Each subtype differed only slightly from the most common pattern using each method. PFGE analysis at a 65% cut-off corresponded to the MLST Clonal Complex (CC); PFGE similarity by 85% or above corresponded to the same MLST Sequence Type (ST). Strain relationships determined by *spa*-typing were likewise concordant with MLST ST designation. PFGE and *spa*-typing could therefore be used as frontline typing systems for multicentre surveillance of MRSA and most members of HARMONY are also members of the *spa*-typing network "SeqNet" [10].

From this work, SCC*mec*, together with MLST was recommended by the HARMONY group to characterise MRSA clones [8]. However, several countries still wanted to use their own names for their strains [8]. In Table 3 examples of nomenclature used in UK are listed and many more are now described (see the utility section below). Experience with *spa*-typing has grown since the project started [11], although for countries with fewer circulating strains its reduced discrimination compared with PFGE is a disadvantage and sequence typing of other genes will most probably be needed [12,13]. Its major advantages over PFGE are ease of interpretation, automation, speed and ability to export results between centres. There is some concern that occasional "violations" of MLST CC assignment by *spa*-typing [14] can occur and so various groups are examining additional genes [12,13]. At present, *spa*-typing may be complemented by the use of additional techniques such as PFGE, MLST, SCC*mec*. This may be supplemented with toxin gene or *agr*-typing depending on the epidemiological or other questions that are being posed and the strains present in a country. International work is underway at standardising the SCC*mec* approaches and this will further increase the discrimination of the techniques, although robust validation will be required.

Table 3

Examples of multi-country clones of methicillin-resistant *Staphylococcus aureus* (MRSA)

HARMONY MRSA nomenclature: MLST Clonal Complex (CC) ; SCC*mec* Designations	Countries and exemplar of English EMRSA nomenclature
MLST CC 5; SCC*mec* I	Belgium, Finland, Germany, Slovenia, Poland, UK; EMRSA-3*
MLST CC8; SCC*mec* IV	Belgium, Finland, France, Germany, Greece, Ireland, Poland, Slovenia, Spain, Sweden; "Iberian Clone"
MLST CC22; SCC*mec* IV	Belgium, Finland, Germany, Ireland, ,Sweden, The Netherlands, UK; EMRSA-15*
MLST CC30; SCC*mec* II	Australia, Belgium, Finland,The Netherlands, Sweden, UK; EMRSA-16*
MLST CC45; SCC*mec* IV	Belgium, Finland, Sweden

* Countries have national names for many of these strains, those for the UK are listed here with an*.
See reference [8] for further details of PFGE and *spa* typing examples; EMRSA = Epidemic methicillin-resistant *Staphylococcus aureus*

Utility of the HARMONY PFGE database

Several countries found the HARMONY experience particularly timely. The PFGE database and protocol was made publicly accessible at: http://www.harmony-microbe.net/microtyping.htm (last accessed 10 April 2008) and has been used by many people from within and outside the EU. In Sweden, the isolates provided made it possible to build a national MRSA-PFGE-database in 2000. It included PFGE patterns of a selection of HARMONY strains and compared, consecutively, incoming PFGE patterns of all Swedish MRSA isolates. Awaiting an international consensus on PFGE pattern nomenclature (which we proposed but did not achieve with other IUMS centres), the Swedish database drew on the HARMONY pattern designations used at the time, adding Swedish designations

when needed [15]. The Swedish MRSA database, including PFGE patterns, normalised against *S. aureus* NCTC 8325, as well as *spa* types (from 2006 and onwards) and MLST STs, providing a national overview, and facilitated exchange of data with laboratories around the world.

Finland established a similar database in 2000, with PFGE still used as the initial typing approach [16]. Interestingly, the lack of transfers of patients between cities in Finland until 2000 was a major factor contributing to MRSA being more contained in this country [16]. Increasingly, patients in many countries are travelling between cities for treatment, either because they think they can get better service elsewhere [17], or because the procedures prescribed are not available in their own city hospital [16,17]. There is also an increased exchange of patients between nursing homes and hospitals, with MRSA increasingly spreading within these healthcare establishments [16,17]. It is therefore plausible to ask whether these factors could perhaps explain the more recent spread of MRSA between cities in Finland [18], as happened earlier in the case of epidemic MRSA-16 in the UK (UK EMRSA-16) [19].

In an impressive initiative, Denmark collaborated with Sweden and Finland to compare MRSA isolated in these three Nordic countries during 2003-2004, again including the HARMONY strains in the comparisons [20] and utilising the HARMONY PFGE protocol.

Several countries with a low incidence of MRSA experienced importation of epidemic MRSA from endemic MRSA countries. The HARMONY database enabled them to confirm that these MRSA strains were indeed indistinguishable from those described in their countries of origin. This enabled the international community to reflect on how the same MRSA strains were behaving in different healthcare settings and patient types. A recurring observable fact in these situations was the rapid spread of these epidemic MRSA strains on affected wards. Some of the infection control teams commented to HARMONY centres that it was far in excess of what they had encountered previously. Audits of infection control in these countries found that the spread was particularly prominent in places where hand hygiene was poor and there were also comments stating that excessive workloads and sub-optimal staffing had been a major driver.

Coagulase negative staphylococcal quality assurance exercise

In 1999, seven of the HARMONY participating laboratories requested another external quality assurance exercise for coagulase negative staphylococci (CNS). Three centres were already considering adopting the new HARMONY PFGE MRSA typing protocol to type CNS and they wanted to know if its discriminatory power was sufficient. For CNS the commonest epidemiological problem is exploring whether pairs of isolates (e.g. isolates from the bloodstream and an intravenous canula from the same patient) are distinguishable. Comparisons are thus needed on the same gel rather than several different gels, as is often the case for MRSA typing referrals. The central laboratory thus sent out 12 isolates of four different species to these seven laboratories in a blinded manner. These included two pairs of duplicate isolates. The results were interpreted in each laboratory, and also objectively in a software program by the coordinating centre. The results were quite remarkable, in that only one centre failed to identify exactly two isolates (a one band difference between two of the isolates probably due to poor gel staining). In addition, the HARMONY protocol proved to be at least equal to the various in-house CNS typing PFGE protocols. This was an important finding, in that the use of a single protocol for all staphylococci would facilitate training, avoid potential confusion and enable inter-centre comparisons, should these be necessary (e.g. exploration of multi-antibiotic-resistant CNS outbreaks following the transfer of patients between different specialised paediatric care (including neonatal) units).

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