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SCIENTIFIC REPORT OF EFSA

Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* in food-producing animals and food [1]

European Food Safety Authority[2,3]

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ABSTRACT

In this report, proposals to improve the harmonisation of monitoring of prevalence, genetic diversity and antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) from food-producing animals and food derived thereof by the European Union Member States are presented. The primary route of zoonotic transmission of MRSA is considered to be the direct or indirect occupational contact of livestock professionals with colonised animals, while the role of food as a source of human colonisation or infection is presently considered to be low. Sampling recommendations have therefore prioritised several different food-producing animal populations previously described as MRSA reservoirs and, to a lesser extent, food produced by these animals. Monitoring in primary production, including at slaughter, is pivotal because of the main transmission route, while additional monitoring in food may help with the assessment of consumers' exposure via this route. A consistent monitoring in broiler flocks, fattening pigs and dairy cattle, as well as in veal calves under 1 year of age and fattening turkey flocks, in those countries where production exceeds 10 million tonnes slaughtered/year, is recommended every third year on a rotating basis. It is proposed that breeding poultry flocks and breeding pigs, as well as meat and raw milk products, are monitored on a voluntary basis. Representative sampling should be made within the framework of the national *Salmonella* control programmes for the poultry populations targeted, at the slaughterhouse for calves and either on farm or at the slaughterhouse for fattening pigs. Harmonised analytical methodologies for identification, typing and further characterisation of MRSA are proposed. The use of the microdilution method applied to a harmonised set of antimicrobials, and interpreted using EUCAST epidemiological cut-off values for antimicrobial susceptibility testing of MRSA, is recommended. Finally, full support is given to collection and reporting of isolate-based data, in particular to enable analysis of multi-resistance.

KEY WORDS

Harmonisation, monitoring, reporting, antimicrobial resistance, methicillin-resistant *Staphylococcus aureus*

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SUMMARY

Directive 2003/99/EC[4] on the monitoring of zoonoses and zoonotic agents obliges the European Union Member States to collect relevant and, where applicable, comparable data on zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks. In addition, Member States shall monitor the zoonotic agents and the sources of disease outbreaks in their territory, and assess trends, and transmit to the European Commission a report covering the data collected every year. Data collected in the framework of Directive 2003/99/EC relate to the occurrence of zoonotic agents isolated from food-producing animals, food and feed, as well as to antimicrobial resistance in these agents. Also foreseen is the possibility of broadening the scope of the antimicrobial resistance monitoring to other zoonotic agents in so far as they present a threat to public health.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is generally resistant to beta-lactam antimicrobials, such as all penicillins, cephalosporins and carbapenems. MRSA colonisation in production animals detected in recent years has in several cases resulted in infections in humans, and infections with livestock-associated strain of MRSA may today be considered as a zoonosis. Pigs, in particular, have been acknowledged as an important source of colonisation with livestock-associated MRSA in pig farmers, veterinarians, and their families, through direct or indirect contact with pigs. In order to increase awareness and to assess the occurrence of MRSA in pig primary production across the EU, an EU-wide baseline survey was performed in 2008 to obtain comparable preliminary data on the occurrence and diversity of MRSA in pig primary production in all Member States through a harmonised sampling scheme. MRSA has since been detected in cattle, chickens, horses, pigs, rabbits, seals, cats, dogs and birds. An assessment of the public health significance of MRSA in animals and food was issued by the European Food Safety Authority in 2009.

The European Food Safety Authority received a mandate from the European Commission to assess whether, in light of the experience accrued with the production of the European Union Summary Reports on Antimicrobial Resistance, the latest scientific opinions issued by the European Food Safety Authority on the issue of antimicrobial resistance and efforts to increase the comparability between findings from the food and animal sector and those gathered in the humans, there is a need to revise existing technical specifications on the harmonised monitoring of antimicrobial resistance in several food-producing animal populations and derived food. In response, the European Food Safety Authority published a first scientific report on the "Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food" on 14 June 2012. The current report provides an extension to cover the harmonised monitoring and reporting of comparable prevalence, characterisation and antimicrobial susceptibility data on MRSA from food-producing animals and food.

Until now the primary route of zoonotic transmission of livestock-associated MRSA has been considered to be the occupational contact of livestock professionals with animals harbouring this type of MRSA. Therefore, monitoring the occurrence and diversity of MRSA in primary production, including at slaughter, seems pivotal, while monitoring in food may also help with the assessment of consumers' exposure via this route, although to date this route of transmission has been deemed of minor importance. In addition, antimicrobial susceptibility data on MRSA isolates are useful in directly informing on the emergence of strains of potential public health significance, but can also provide important epidemiological information on the spread of particular strains between the animal and human populations, particularly when investigated in conjunction with molecular typing data.

In the current report, sampling specifications, including the frequency and recommended location of sampling, are provided for several different types of production animals and food derived thereof.

[4] Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. OJ L 325, 12.12.2003, p. 31–40.

Consistent monitoring of potential livestock-associated MRSA reservoirs previously described, such as broiler flocks, fattening pigs and dairy cattle, as well as veal calves under 1 year of age and fattening turkey flocks in those countries where production exceeds 10 million tonnes slaughtered/year, is recommended every third year on a rotating basis. It is also proposed that animal populations, such as breeding flocks of *Gallus gallus* (meat sector), breeding flocks of turkey and breeding pigs, which may play a role in the epidemiology of MRSA (potential clonal diffusion) in the production sector in question, are monitored on a voluntary basis. Beef cattle and horses may be included in the voluntary monitoring. Representative sampling should be performed within the framework of the national *Salmonella* control programmes for the poultry populations targeted and at the slaughterhouse for calves. Dairy farms can be monitored through bulk tank milk sampling. It is proposed that fattening pigs are sampled at the slaughterhouse in countries where the prevalence of MRSA in pigs is low or which have little knowledge of MRSA situation, while on-farm monitoring is suggested for countries where there is a proven important prevalence of MRSA in pigs and a desire to estimate farm-level prevalence and better assess the epidemiology of MRSA. MRSA monitoring may also be carried out on a voluntary basis in the following food categories: (1) broiler meat, turkey meat, pork, beef and veal, either at the cutting/transformation plant or at the retail level; and (2) raw milk and raw milk products at the dairy/processing plant or at retail level. Sample size is greatly affected by the epidemiological situation and the purpose of sampling, therefore, it should be calculated at the MS-level. With regard to the minimum MRSA isolate sample size for monitoring antimicrobial susceptibility, the figure of 170 isolates per year is recommended as an optimal isolate sample size, although this number of isolates may be difficult to achieve in food production sectors with medium to low MRSA prevalence. In that latter case, a minimum number of samples is proposed to be collected enabling to check that the prevalence is not above an expected level. Example calculations for several possible values are given in the respective section of the report.

Harmonised analytical methodologies for identification, typing and further characterisation of MRSA are proposed. They comprise the following steps: isolation of presumptive MRSA (including pre-enrichment and selective enrichment steps) and confirmation of MRSA by detecting notably the presence of *mecA* or *mecC* using preferably multiplex PCR or, in isolates negative for these genes, phenotypical testing for resistance to cefoxitin. Confirmed MRSA isolates are further *spa*-typed in order to determine the corresponding clonal complex. Isolates in which no clonal complex can be determined based on the *spa*-type should be multilocus sequence typed. Further analytical tests, such as SCC*mec* typing, pulsed field gel electrophoresis, micro-array for virulence and other genes, and whole-genome sequencing, can be performed to further characterise isolates. Molecular typing and phenotypic information should be used to investigate the occurrence of shared types of MRSA occurring in different epidemiological niches.

The use of a microdilution method applied to a harmonised set of antimicrobials and accompanied by the application of European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values as interpretative criteria of resistance for antimicrobial susceptibility testing of MRSA is recommended. Two lists of antimicrobial substances are proposed, a recommended set and an optional set. Optimal, advised and minimum concentration ranges to be tested have also been proposed. Both the EUCAST epidemiological cut-off values and the clinical breakpoints are, however, included in the recommended range, so that the data can be easily compared with those of human isolates. Finally, full support is given to the collection and reporting of isolate-based data, in order to enable more in-depth analyses to be conducted, in particular on the occurrence of multi-resistance.

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BACKGROUND AS PROVIDED BY EC

In accordance with Directive 2003/99/EC on monitoring of zoonoses and zoonotic agents, Member States must ensure that monitoring provides comparable data on the occurrence of antimicrobial resistance (AMR) in zoonotic agents and, in so far as they present a threat to public health, other agents. In particular, Member States must ensure that the monitoring provides relevant information at least with regard to a representative number of isolates of *Salmonella* spp., *Campylobacter jejuni* and *Campylobacter coli* from cattle, pigs and poultry and food of animal origin derived from these species.

Commission Decision 2007/407/EC[5] implementing Directive 2003/99/EC, lays down detailed and harmonised rules for the monitoring of AMR in *Salmonella* in poultry and pigs. The technical specifications of this Decision are applicable until the end of 2012.

Control of AMR is a high priority for the Commission, which issued a Communication to the European Parliament and the Council on a 5-year action plan to fight against AMR in the European Union (EU) that was adopted on 17 November 2011. In order to follow trends on AMR in zoonotic agents and to evaluate the results of the strategy, new implementing provisions on AMR monitoring in Directive 2003/99/EC must be considered.

In 2007 and 2008 the European Food Safety Authority (EFSA) Task Force on Zoonoses Data Collection endorsed reports including guidance for harmonised monitoring and reporting of AMR in *Salmonella*, *Campylobacter* and commensal *Escherichia coli* and *Enterococcus* spp. from food animals. These reports provided the technical, science-based input for the detailed rules on AMR monitoring which are in force until the end of 2012.

In the meantime, EFSA's Panel on Biological Hazards has adopted several opinions on AMR in zoonotic agents such as:

- The Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum beta-lactamases and/or AmpC beta-lactamases in food and food-producing animals adopted on 7 July 2011;
- Joint Opinion on AMR focused on zoonotic infections adopted on 28 October 2009;
- Assessment of the Public Health significance of methicillin resistant *Staphylococcus aureus* (MRSA) in animals and foods, adopted on 5 March 2009;
- Food borne antimicrobial resistance as a biological hazard, adopted on 9 July 2008.

In addition, EFSA has published several reports on AMR monitoring in zoonotic agents in the EU such as:

- European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union in 2009, approved on 29 April 2011;
- The Community Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union in 2008, approved on 15 June 2010;
- The Community Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union in 2004-2007, approved on 28 February 2010.

The Commission would like to review the monitoring requirements for AMR in zoonotic agents. Before doing that, it would be useful to consider the need for updates to the 2007 and 2008 EFSA reports taking into account the most recent scientific opinions on AMR, technological developments, recent trends in AMR occurrence and knowledge on consequences for human health.

[5] Commission Decision of 12 June 2007 on a harmonised monitoring of antimicrobial resistance in *Salmonella* in poultry and pigs. OJ L 153, 14.6.2007, p. 26–29.

TERMS OF REFERENCE AS PROVIDED BY EC

In accordance with Article 31 of Regulation (EC) No 178/2002[6], EFSA is requested to provide scientific and technical assistance proposing updates, where relevant, to the 2007 and 2008 EFSA reports on harmonised monitoring and reporting of methicillin resistant *Staphylococcus aureus* (MRSA) from food-producing animals and food. Comparability with results from human monitoring should also be ensured. In particular EFSA should:

1. Provide detailed guidance on the monitoring of MRSA: food animal species and/or foodstuffs and methodologies which should be considered as most relevant for antimicrobial resistance (AMR) monitoring from a public health perspective, taking into account AMR mechanisms;

2. Reconsider the antimicrobials, epidemiological cut-off values and recommended optimum concentration ranges to be used for susceptibility testing of MRSA isolates;

3. Indicate the best format for the collection and reporting of data.

[6] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 01.02.2002, p. 1–24

CONSIDERATION/SCIENTIFIC REPORT

1. Introduction

Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents obliges the European Union (EU) Member States (MSs) to collect relevant and, where applicable, comparable data on zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks. In addition, MSs shall monitor sources of these agents and outbreaks in their territory, and assess trends, and transmit to the European Commission (EC) a report covering the data collected every year. The data transmitted under Directive 2003/99/EC relate to the occurrence of zoonotic agents isolated from animals, food, and feed, as well as to antimicrobial resistance in these agents. Also foreseen is the possibility of broadening the scope of the antimicrobial resistance (AMR) monitoring to other zoonotic agents in so far as they present a threat to public health.

Methicillin-resistant *Staphylococcus aureus* (MRSA), which is generally resistant to beta-lactam antimicrobials, such as all penicillins, cephalosporins and carbapenems, has been recognised as an important cause of infection in hospitals for several decades; the last two decades have also seen the emergence of strains of MRSA that are particularly associated with community-acquired infections in humans. A development in recent years has been the detection of livestock-associated MRSA (LA-MRSA) in production animals in several MSs, such as lineage multilocus sequence type 398 (ST398). MRSA has since been detected in cattle, chickens, horses, pigs, rabbits, seals, cats, dogs and birds. An assessment of the public health significance of MRSA in animals and food was issued by the European Food Safety Authority (EFSA) in 2009 (EFSA, 2009a).

In particular, pigs, which are frequently carriers of methicillin-sensitive *Staphylococcus aureus* (MSSA) ST398 (Hasman et al., 2010), have been recognised as a source of MRSA colonisation among pig farmers, veterinarians, and their families, through direct or indirect contact with pigs. MRSA ST398 has therefore been considered an occupational hazard for humans. In order to increase awareness and to assess the occurrence of MRSA in pig primary production across the EU, an EU-wide baseline survey was performed in 2008 to obtain comparable preliminary data on the occurrence and diversity of MRSA in pig primary production in all MSs through a harmonised sampling scheme (EFSA, 2010). Pooled dust samples collected from pig holdings were tested for MRSA and all isolates were subjected to *spa*-typing and determination of their MRSA ST398 status. The survey results indicated that MRSA was common in breeding pig holdings in some MSs, while in other MSs the prevalence was low (EFSA, 2009b). MRSA ST398 was by far the most predominant MRSA lineage identified. Further investigation of the diversity of MRSA *spa*-types also showed that the distribution of *spa*-types differed significantly between countries. MRSA isolates not belonging to ST398 were detected in six MSs. In these MSs, the MRSA *spa*-types isolated varied, although the t011 *spa*-type was by far the most common. MRSA *spa*-types not belonging to ST398 described in human medicine were also detected among the surveyed pig holdings. The EU-wide baseline survey also revealed preliminary factors associated with MRSA contamination of holdings with breeding pigs, such as herd-size and pig trade contacts, which have since been confirmed as risk factors/indicators (Broens et al., 2011a; Ciccolini et al., 2012). The strong pyramidal structure of the swine-production chain, in which there is a predominant flow of animals from a few breeding herds to numerous production holdings, may facilitate the vertical dissemination of MRSA between the breeding and production holdings.

The recognised LA-MRSA strain, which appears to be primarily acquired by occupational exposure, can on occasion be introduced into the general community and/or hospitals. It is also important to distinguish between the epidemiology of MRSA in relation to production animals and companion animals, which generally are infected with classical human variants of MRSA (Manian, 2003; Weese et al., 2006). Indeed, food-producing animals are not the only source of zoonotic MRSA infections in humans: direct contact with companion animals, for example dogs, cats and horses, may also play a role. However, according to the mandate received, these technical specifications focus on food-producing animals and food thereof.

Currently, there are no harmonised definition of MRSA or recommendations for the monitoring of MRSA in animal populations and food in the EU, although a small number of MSs carry out monitoring consistently. The recent detection of a strain of MRSA carrying a novel *mecA* gene that eludes detection by conventional PCR tests requires a revision of the current definition of MRSA, as such MRSA isolates are misidentified and their prevalence underestimated. The sampling stages investigated, the types of samples taken and the analytical methods used vary from country to country and also between investigations. In addition, the most recent European Union Summary Report (EUSR) on AMR shows that a limited number of MSs reported data on MRSA antimicrobial susceptibility (EFSA and ECDC, 2012). There are no EFSA recommendations for the susceptibility testing of *S. aureus* or MRSA, and the reporting MSs applied either breakpoints from the Clinical Laboratory Standards Institute (CLSI) or epidemiological cut-off values (ECOFFs) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to assess the resistance of isolates. To enhance the comparability of results between MSs, it is important to agree upon a harmonised method, a common panel of relevant antimicrobials to test, as well as on standard thresholds for the interpretation of susceptibility. This lack of harmonisation has hampered the analyses of data at EU level and, therefore, there is no clear picture of the occurrence, diversity and susceptibility of MRSA in the relevant animal populations and food categories in the EU. The objectives of these technical specifications are to propose a harmonised methodology to be used in the monitoring of the most relevant production animals and foodstuffs throughout the EU.

This report is the second report addressing a mandate received from the EC on the provision of scientific and technical assistance on the harmonised monitoring of AMR in zoonotic agents. The first scientific report of the EFSA, on the "Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella, Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food" (EFSA, 2012a), was provided under the same mandate and published on 14 June 2012. This scientific report specifically addresses the terms of reference of the mandate regarding MRSA and covers specifically monitoring, collecting and reporting comparable prevalence, diversity and antimicrobial susceptibility data on MRSA from food-producing animals and food under Directive 2003/99/EC. The report provides a rationale and presents the key elements for a harmonised monitoring of prevalence and AMR yielding comparable data. The proposals are based on the thorough review of literature, the EFSA published opinions on MRSA and AMR and the MRSA data reported by MSs in the EUSR covering the period 2008-2010.

2. Rationale for the choice made for the monitoring of MRSA proposed

2.1. Rationale for the definition of MRSA

MRSA typically acquires resistance to methicillin (and most other beta-lactam antimicrobials) through possession of the *mecA* gene, which encodes an altered penicillin-binding protein PBP2' (or PBP2a) that does not bind most penicillins or cephalosporins (Hartman and Tomasz, 1984). Some strains of *S. aureus* possess an alternative mechanism of resistance, attributable to hyperproduction of the *S. aureus* beta-lactamase enzyme, which hydrolyses the beta-lactam ring of penicillin and cephalosporin compounds, inactivating them (Brown et al., 2005).

Recently, a novel *mecA* homologue (with approximately 70 % similarity to the *mecA* gene) that also confers methicillin resistance was identified in *S. aureus* isolates from dairy cattle and humans in the United Kingdom and in France, and from humans in Denmark. This has been designated *mecC*[7]; *mecC* occurs in a previously unidentified genetic element, which has been designated SCC*mec XI* (García-Álvarez et al., 2011; Laurent et al., 2012; Paterson et al., in press). The novel *mecA* homologue has been confirmed in an archived human *S. aureus* isolate from 1975 from Denmark and has also been described in humans in Ireland (Shore et al., 2011) and Germany (Cuny et al., 2011). Isolates of *S. aureus* carrying the novel *mecA* element have not, until recently, been detected by most methods currently employed to detect "classical" MRSA. They have been associated with clinical disease in both cattle (mastitis in dairy cows) and humans. To date *S. aureus* isolates carrying the novel *mecA* homologue have been found to belong to either clonal complex 130 (CC130) or sequence type 425 (ST425) (García-Álvarez et al., 2011; Shore et al., 2011). The observation that most previously reported CC130 isolates are from bovine sources has been considered to suggest that CC130 isolates are of bovine origin (Shore et al., 2011).

In consequence, for the purpose of the harmonised monitoring of MRSA in animals and food in the EU, the following definition of MRSA is proposed: *S. aureus* harbouring either the *mecA* or the *mecC* genes or, if negative for these genes, phenotypically resistant to cefoxitin.

2.2. Rationale for the choice of the objectives of monitoring MRSA prevalence and diversity

- **Rationale for monitoring occurrence and diversity of MRSA in animals and food**

To date, the primary route of zoonotic transmission of these bacteria has been considered the occupational contact of livestock professionals with animals harbouring LA-MRSA (Bisdorff et al., 2012). The EU-wide baseline survey carried out in holdings with breeding pigs highlighted substantial differences in the prevalence of MRSA and in the diversity of the non-ST398 MRSA in the breeding pig populations of the MSs (EFSA, 2009b). Since the conduct of the EU-wide baseline survey in pigs, numerous studies aiming to assess the prevalence and the diversity of MRSA have been carried out in various food-producing animal populations in a number of MSs. They have shown that MRSA not only occur in herds of pigs but are also prevalent in the different populations of cattle (veal calves, beef and dairy cows) (García-Álvarez et al., 2011; Spohr et al., 2011; Kreausukon et al., 2012) and of poultry (broilers and laying hens of *Gallus gallus* and turkeys) (Nemati et al., 2008; Mulders et al., 2010). Moreover, MRSA is increasingly being detected in animal-derived foods. Although many of these studies are cross-sectional, some of them suggest an increase in the prevalence of MRSA in pigs or cattle.

Most of the isolates detected in the course of these studies are assigned to one clonal complex (CC), the so-called livestock-associated CC398. However, it has been pointed out that, especially in poultry, other strains are also prevalent, such as CC9 in broilers and CC5, most notably in turkeys. Important diversity of MRSA strains has been also recorded among similar production lines in different MSs. For example, in Italy, isolates of Multi Locus Sequence Type (MLST) ST1 have frequently been detected in the framework of the EU-wide baseline survey in breeding pigs. The *spa*-type t108,

[7] Previously denoted as $mecA_{LGA251}$.

frequently observed in the Netherlands, is less frequent in other neighbouring MSs; conversely, the *spa*-type t034, which is very common in northern Germany, is less frequently detected in the neighbouring provinces of the Netherlands.

LA-MRSA may pose a hazard to human healthcare systems because of the risk that colonised livestock professionals will introduce into healthcare facilities and/or cause spread into the community of emerging MRSA strains of particular virulence. However, to date, it seems that the capacity for dissemination in humans (patient-to-patient transmission) of LA-MRSA, in particular ST398, is low compared with hospital-associated MRSA. Conversely, community-acquired MRSA strains (CA-MRSA) may also spread from the human community to production animal sectors, in which they may diffuse, multiply and evolve further. Recent results on the gene pool of MSSA CC398 suggest that this strain could originate from humans (Price et al., 2012).

MRSA has been identified in numerous types of meat (de Boer et al., 2009; Tenhagen et al., 2011), in raw milk and raw-milk products, and it is considered that the presence of MRSA in food may be associated with a risk of introduction of the bacteria into households. However, the role of food as a source of human colonisation or infection with MRSA is presently considered to be minor, since epidemiological studies have shown that LA-MRSA is fairly infrequent among people without direct or indirect contact with livestock, who cannot be exposed other than through food or the environment (Bisdorff et al., 2012). The transmission of LA-MRSA infection by food, in particular fresh meat, has been recognised to be very rare (EFSA, 2009a), and food has not been considered an important source of LA-MRSA in human colonisation. However, MRSA have been shown to evolve continuously, and changes in characteristics, such as virulence and transmissibility, may most likely occur in the future. Therefore, regular monitoring would seem to be advisable to identify the subtypes of MRSA that are prevalent.

- **Rationale for monitoring antimicrobial susceptibility of MRSA in animals and food**

MRSA antimicrobial susceptibility data are likely to be useful in assessing the potential impact of the use of antimicrobials in animals and the public health implications of certain isolates. However, susceptibility testing may also be important in the presumptive identification, routine detection and monitoring of spread of particular clones of MRSA, particularly when combined with certain other molecular typing data, such as *spa*-type or Panton-Valentine leukocidin (PVL) toxin status. For example, data presented from Switzerland in the EUSR on AMR for 2010 showed that isolates belonging to the most commonly detected genotype, ST398-t034-V, had an identical resistance profile, except for one isolate which was susceptible to streptomycin. Although it is important to note that the detection of certain types of resistance, especially glycopeptide resistance, in *S. aureus* is problematic (Brown et al., 2005), particular MRSA clones may have susceptibility characteristics which can assist in their identification. As a further example, in the United Kingdom, strains EMRSA-15 (CC22) and EMRSA-16 (CC30) emerged as epidemic human strains in the 1990s and are both usually resistant to ciprofloxacin and macrolides. Isolation media containing ciprofloxacin have therefore been developed and used for selective isolation of these strains, where they are prevalent (Brown et al., 2005; Ellington et al., 2010).

Moreover, differences in the antimicrobial resistance of MRSA strains from different sources have been observed, with isolates from healthcare settings being mostly resistant to ciprofloxacin, an antimicrobial of the fluoroquinolones class, whereas LA-MRSA are frequently susceptible to this class of antimicrobials. In addition, among livestock populations differences between strains with respect to AMR have been observed, with non-CC398 strains being more frequently resistant to ciprofloxacin than CC398, and t034 showing a different resistance pattern from the most frequent *spa*-type, t011 (Tenhagen et al., 2009; Schroeter and Käsbohrer, 2012).

- **Objectives and approach of MRSA monitoring**

Prevalence and characteristics of LA-MRSA in animals and food may evolve, and regular monitoring is therefore required to detect changes in prevalence and the emergence of new subtypes of MRSA,

possibly displaying particular virulence characteristics. It is acknowledged that an MRSA monitoring programme in production animals and food thereof should primarily assess the diversity of prevalent MRSA strains to allow detection of the emergence of strains of particular virulence and constant comparison with the MRSA strains prevalent in humans. Secondarily, monitoring MRSA should also enable to assess MRSA prevalence in different epidemiological units of interest (e.g. animals, flocks, farms, depending of the production sector in question), and to follow-up trends over time. In addition, where MRSA prevalence is recorded as high, the follow-up of negative units may be of interest to monitor for protection, to search for risk factors for infection and to assess spread from positive units. Considering that occupational contact with live farm animals is currently the predominant route of transmission of LA-MRSA, monitoring in primary production, including at the slaughterhouse, seems pivotal. However, monitoring in food may also help assess the risk of infection of consumers via this route, although at present this route is considered of minor importance.

Moreover, comparison of MRSA occurring in different ecosystems, such as humans and various animal populations, may be of significant value for estimating the different influences on different ecosystems, for example for assessing the relative importance of various factors on the emergence and spread of AMR. In addition, and probably more importantly in the case of LA-MRSA, this may also reveal links between the different ecosystems and, therefore, may help to exclude or to infer connections where the prevalent subtypes and resistance profiles are either different or similar in the two populations. To maximise the cost-effectiveness of monitoring, it is suggested that in the first instance MRSA isolates should be investigated using *spa*-type, where necessary MLST type, PVL toxin status and antimicrobial resistance profile. This provides a great deal of key information and in particular provides monitoring for the possible incursion of the recognised community-acquired PVL–positive human strains of MRSA into food-producing animals. Supplementary tests such as micro-array analysis should be performed on isolates for virulence gene screening as appropriate and in a targeted way to further investigate the epidemiology of MRSA.

2.3. Rationale for the choice of the animal populations to be monitored

Since 2003, an increasing number of studies have reported the prevalence of a specific strain of LA-MRSA, MRSA ST398, in food-producing animals and food derived from these animals (van Rijen et al., 2008; Mulders et al., 2010). Since the EU-wide baseline survey in holdings with breeding pigs in 2008-2009, numerous studies aiming to assess the prevalence and diversity of MRSA have been carried out in various food-producing animal populations in a number of MSs. They have shown that LA-MRSA occur not only in herds of pigs but also in cattle (veal calves, beef and dairy cows) and poultry (broilers and laying hens of *Gallus gallus* and turkeys).

2.3.1. Pigs

It is proposed that fattening pigs are mainly targeted because they account for a large share of the overall pig population. The EU baseline survey carried out in 2008 showed that a great diversity of MRSA strains can be found in the breeding pig population, and these strains are likely to be transmitted to fattening pigs; thus, the situation in fattening pigs will to some extent mirror that in the breeding pig population. In a recent study of a representative sample of 50 randomly selected pig farms in Belgium, 68 % (34 farms) tested positive for MRSA (defined as at least one sample per farm testing positive for MRSA) (Crombé et al., 2012). Open farms were found to have higher among-farm and within-farm prevalence of MRSA compared to closed farms, while within closed farms piglets had a higher MRSA prevalence compared to sows and fattening pigs (Crombé et al., 2012). In line with this, a German study found that fattening farms buying pigs from several sources were at higher risk of being positive for MRSA than farms producing their own piglets or farms buying from only one or two sources (Alt et al., 2011). This implies that studying the fattening pig population is likely to be more sensitive for detecting MRSA in the pig population. However, it is known that occupational exposure is more pronounced in farms with breeding pigs due to the more intensive handling of sows and piglets as compared with farms raising fattening pigs. Therefore, monitoring of MRSA in those herds on a voluntary basis is also recommended.

2.3.2. Cattle

To date, MRSA prevalence in dairy farms has been recorded to be lower than that in veal calf-rearing facilities (Graveland et al., 2010). In a recent representative study in Germany, the prevalence of MRSA infection among dairy herds, based on testing of bulk tank milk samples, was 4.4 % (Kreausukon et al., 2012), and regional studies performed in southern Germany reported a comparable, while slightly lower, prevalence of 2.2 % (Friedrich et al., 2011). Likewise MRSA has been shown to occur in dairy herds in other MSs such as Belgium and also in other parts of the world (Haran et al., 2012).

By contrast, a study of 102 randomly selected veal calve farms revealed that 88 % of the farms investigated housed at least one animal testing positive for MRSA and overall 30 % of animals were positive for MRSA (Graveland et al., 2010). At the slaughterhouse level, a study in Germany, in 2009, found that the prevalence of MRSA among veal calves at stunning was 35 % (Tenhagen et al., 2011). Little is known about the persistence of MRSA in veal calf-rearing facilities over consecutive production rounds (EFSA, 2009a). Beef animals have only recently been targeted by the national MRSA monitoring programme in Germany. However, the limited data available to date on the prevalence of MRSA in these animals at the slaughterhouse seem to show a considerably lower occurrence as compared with that assessed in veal calves (B.-A. Tenhagen, Bundesinstitut für Risikobewertung, Germany, personal communication, 2012). By contrast, in a survey performed in Denmark in 2010, 192 cattle animals - mainly young bulls - from at least 174 different farms were sampled by skin swabbing at the slaughterhouse and all tested negative for MRSA (DANMAP, 2010). Similar data have been reported from Canada. It can therefore be assumed that, among the different cattle production lines, veal calves have the highest MRSA burden and thus it is recommended that monitoring of MRSA in cattle primarily targets veal calve populations. Nevertheless, as the dairy cow population is the basis for the veal calve production and MRSA have been detected in bulk tank milk, dairy herds should also be monitored.

2.3.3. Poultry

The monitoring of MRSA in poultry should focus chiefly on broilers of *Gallus gallus* and fattening turkeys, as these constitute the main poultry populations in the EU and carcasses of these animals may be substantially contaminated by MRSA at the slaughterhouse. In a Belgian survey conducted in 2007, animals from 14 broiler farms and 10 laying hen farms were examined. MRSA was found in broilers from 2 of the 14 farms but was not found in any of the samples originating from laying hen farms (Persoons et al., 2009). In another Belgian study, conducted in 2006, healthy chickens were sampled from 39 randomly selected farms and were examined for MRSA. Chicken from five of those farms tested positive for MRSA (Nemati et al., 2008). A regional study in Germany detected MRSA in 18 out of 20 fattening flocks of turkeys and in the personnel attending the animals (Richter et al., 2012). In a national survey, 19.6 % of the tested turkey flocks harboured MRSA (http://www.bvl.bund.de/SharedDocs/Downloads/01_Lebensmittel/04_Zoonosen_Monitoring/Zoonosen_Monitoring_Bericht_2010.pdf?__blob=publicationFile&v=6, online). A Dutch study demonstrated transmission of MRSA from broilers to humans dealing with the live birds (Mulders et al., 2010), emphasising the need to monitor broilers as a potential source of MRSA in humans.

2.4. Rationale for the choice of the food categories to be monitored

MRSA have been identified in numerous types of meat (de Boer et al., 2009; Tenhagen et al., 2011). The highest detection rates have been observed in poultry meat. However, MRSA have also been found in meat from pigs, including minced meat, albeit at lower rates than in poultry meat. In some MSs, meat from pigs is also consumed raw in specific meat products. Among bovine meat, MRSA is most often found in veal.

In addition to meat, raw milk has been shown to contain MRSA. With few exceptions, heat treatment of milk is mandatory before marketing. Pasteurised milk, intensively heat-treated milk as well as milk-based products derived from these types of milk are a very unlikely source of consumer exposure to

MRSA and, therefore, are not considered relevant for inclusion in a consistent LA-MRSA monitoring programme. However, raw milk and derived raw milk products, which may be contaminated with MRSA, could be monitored in those MSs where consumption of these products is frequent.

Since the risk of transmission of MRSA to humans through food is considered to be minor, monitoring of meat (from broilers, turkeys, pigs, beef and veal), raw milk and raw milk products could be performed on a voluntary basis in the interested MSs.

2.5. Rationale for the stage of the food chain to be monitored

The choice of which stage in the food chain to monitor is dependent on several unequal considerations. Three stages are typically considered: (1) on farm, where animals or their immediate rearing environment, through secondary samples such as dust or environmental faeces, may be sampled; (2) at the slaughterhouse, where animals held in lairage pens or carcasses may be sampled.

Monitoring MRSA on farm has the main advantage of providing information on the food chain stage that can be best influenced by countermeasures aiming at reducing the development of AMR and the spread of MRSA in production sectors concerned. It is possible to analyse risk factors/indicators and possibly associate MRSA levels with certain farm management practices. Moreover, recent studies in Germany have shown that MRSA may be detected in the vicinity of animal barns (Schulz et al., 2012). MRSA were also detected in farmhouses, pointing to a potential transfer from the barns to the residential area (Geenen et al., 2012). A disadvantage of sampling at the farm level is the higher costs compared with sampling at slaughterhouses.

In most MSs, monitoring MRSA at the slaughterhouse is comparatively more cost-effective to determine prevalence, particularly in the case of low to very low prevalence, or to assess the diversity of the MRSA subtypes prevalent in a production sector, as it has been demonstrated to be highly sensitive. A drawback relates to difficulties in interpreting the prevalence data, as cross-contamination is known to occur during transport and lairage, making it difficult to infer the original MRSA prevalence of the animals on farm (Broens et al., 2011b). Linking the MRSA strains discovered at the slaughterhouse to any particular farm will also be complex, if at all possible. If data on the within-batch prevalence of MRSA are not needed and between-batch comparison is enough, then this disadvantage is less critical.

2.6. Rationale for the choice of the samples for monitoring MRSA

2.6.1. Monitoring MRSA in animals

MRSA have been isolated in various animal production sectors by means of different samples, which may be of two types: (1) environmental samples such as dust swabs, air samples and boot samples, which do not require contact with animals; and (2) animal samples such as nasal swabs, skin swabs, faecal samples and milk samples, which require handling of individual animals. Samples can be analysed either as single samples or as pools of samples. The advantage of sample pooling is that a larger number of samples can be collected and analysed, making the sampling more representative of the herd/flock/batch without increasing the analysis cost dramatically. In the case of environmental dust swabs, the pooling of samples has been shown to decrease the sensitivity of the method (Broens et al., 2011c), but the pooling of nasal swabs and skin swabs, respectively, has been shown to increase the detection rate (Broens et al., 2011c; Friese et al., 2012). The optimal sampling type depends on the animal species tested, the robustness of the sampling procedure and the purpose.

Environmental sampling is useful to determine if a herd is positive or negative for MRSA. The most common method is to use cloths to swab surfaces in the environment of pig stables. This method was used in the MRSA EU-wide baseline survey in breeding pigs carried out in 2008 (EFSA, 2009b). This method has been shown to be relatively insensitive, especially when samples are pooled (Broens et al., 2011c), and will therefore detect MRSA only in herds with a high prevalence. Other environmental sampling methods, such as air sampling and boot swab sampling, have also been used

in a few studies (Broens et al., 2011c; Friese et al., 2012). Air sampling seems to be useful for sampling dust in pig herds and detects MRSA with the same sensitivity as collecting pools of nasal swabs (Friese et al., 2012; Y. Agersø, National Food Institute, Technical University of Denmark, Denmark, personal communication, 2012), but the method has been tested in only one published and one unpublished study. Moreover, air sampling requires air-sampling equipment. Bootswab sampling has been evaluated in one study and may be useful for sampling MRSA in poultry houses (Friese et al., 2012), as it has been extensively used in the EU for assessing Salmonella status of poultry flocks in the framework of the national control programmes.

Animal sampling has been carried out in several food-producing animal species, including pigs, cattle, poultry, sheep and horses. The method most often used in pigs is the use of nasal swabs: either a single swab per individual or one to several pools of four to six swabs per herd. Recently, the use of 'ear swabs' (swabbing the skin behind the ears of pigs) has been also tested, and the results so far suggest this method to be more sensitive than using a similar number of nasal swabs in pigs (Pletinckx et al., 2012).

2.6.2. Monitoring MRSA in food

In most studies, 25 g of meat was sampled, but also other amounts of meat have been used, such as 10 g (Weese et al., 2010). In the EU, protocols for sampling food for many purposes, including the testing of microbiological criteria according to Regulation (EC) No 2073/2005[8], foresee the use of 25 g of meat. In general, for the isolation of bacteria from meat, it is recommended that the surface of the meat is sampled, preferably including the skin (mainly relevant for poultry), as most bacteria are present on the surface of meat. Meat should always be sampled as close to consumers as possible, and sampling for MRSA in meat could be combined with surveillance of meat in general. In the case of raw milk and raw milk-derived products, it is recommended that 25 mL and 25 g of product, respectively, be sampled. Sampling may be performed at the dairy/processing plant or at retail.

2.7. Rationale for the review after first harmonised monitoring

Scientific insights and methodologies evolve continuously. For this reason it is recommended that these technical specifications be reviewed immediately after the completion of the first round of monitoring is completed. This will add new experience and knowledge in the area of MRSA epidemiology.

The combination of new developments in sampling methodology and further insights in the prevalence of MRSA may enable more cost-effective sampling strategies that yield the same amount of information with less effort. In particular, the effect of pooling of samples and the specific nature of samples, nasal swab, dust, skin swab, faecal or other, should be reviewed to ascertain that the optimal strategy is followed.

In this report a unique analytical method is proposed for the sake of harmonisation and comparability. Possible modifications to the method, including an amended protocol recently described (Johne et al., 2012) or the inclusion of additional targets to the multiplex PCR will first need to be addressed by the EURL on AMR both in terms of optimisation and diffusion of the method.

Similarly, the common set of antimicrobials proposed for harmonised susceptibility testing of MRSA may be revised. The list of antimicrobial substances proposed in this report is not exhaustive but considered as an appropriate minimum at the setting-up stage of a harmonised routine monitoring of MRSA.

Additional substances, such as rifampicin and complementary aminoglycosides, or even biocides, such as zinc, which may co-select for other types of resistance, may be considered in the future. A review

[8] Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. OJ L 338,22.12.2005, 1-26, as amended by Commission Regulation (EC) No 1441/2007 of 5 December amending regulation (EC) No 2073/2005 on microbiological criteria for foodstuff: OJ L 322, 07.12.2007, p. 12–29.

along these lines after the first harmonised monitoring by the MS will allow the adjustment of data collection procedures and will also ensure that any improvements in laboratory methodology be incorporated into the programmes that will be performed in later years.

2.8. Rationale for the comparison with the prevalent human clones of MRSA

The monitoring proposed by EFSA in animals and food will provide descriptive data relating to the occurrence and diversity of MRSA in relevant animal populations and food derived from animals. The MRSA isolates collected and analysed in the monitoring programme will have been characterised in a number of different ways, including *spa*-type, possession of PVL toxin, resistance to a panel of antimicrobials and (optionally) whether clindamycin resistance is inducible. This animal and food MRSA data should be compared with the data available from humans, which may be obtained from community or hospital sources and common types should be highlighted. There is no formal mechanism currently in place at EU level for the comparative analysis of results from man, animals and food or for the assessment of potential epidemiological links when particular types are detected. Particular types of MRSA, such as the CA-MRSA strains with PVL, which are currently increasing in importance in human medicine, should be highlighted and prioritised when such analyses are performed.

3. Recommendations on food animal species and/or foodstuffs to be considered for MRSA monitoring from a public health perspective

3.1. General considerations

The major considerations on which these recommendations are based are presented in sections 2 and 4 of the report. A recommendation on the animal populations and/or food categories to be regarded as a priority in a routine MRSA monitoring is presented in synoptic tables, Table 1 and 2, detailing the corresponding biological samples to be collected and the frequency of the sampling.

It is proposed that MRSA monitoring should primarily be focused on domestic production, so that the relationships between the prevalence, the diversity and the AMR of MRSA and the potential impact of putative risk factors can be further analysed.

The set of animal populations and food targeted by national MRSA monitoring programmes may need to be adapted to varying national circumstances and, in particular, voluntarily expanded by MSs as necessary.

3.2. Animal populations to be monitored consistently for MRSA

In establishing a list of mandatory requirements for monitoring, the greatest benefit may result from focusing on the animal populations displaying a significant MRSA prevalence, which may be considered as LA-MRSA reservoirs. The prevalence, genetic diversity and AMR of MRSA strains may vary significantly between animals of different production types or different production stages (within a pyramidal production sector), reflecting the widely differing treatment regimes, management and hygienic conditions and trade practices encountered. Therefore, MRSA monitoring and reporting should be preferentially structured according to production types/animal populations. The animal populations proposed to be monitored consistently for MRSA correspond to various production types of the main food-producing animal species, such as broilers, fattening turkeys, fattening pigs, dairy cows and veal calves (Table 1).

3.3. Animal populations to be monitored for MRSA on a voluntary basis

The possible inclusion of various breeding populations, such as breeding pigs and poultry, among the animal populations to be monitored is acknowledged as being of interest in certain countries (Table 1). These populations may prove to be a source of clonal spread of MRSA in pyramidal animal production sectors and, additionally, specifically necessitate intensive management activities implying a high risk of occupational exposure.

3.4. Foodstuffs to be monitored for MRSA on a voluntary basis

To date, the greatest risk of transmission of MRSA from food-producing animals to humans has been considered to be direct or indirect contact between infected animals and members of certain occupational groups rather than through food. Thus, the monitoring of MRSA in meat derived from animal populations displaying a significant MRSA prevalence, and to which the consumer will most likely be exposed through food thereof, may be performed on a voluntary basis (Table 2). The monitoring of raw milk and raw milk-derived products is included in this voluntary scheme.

Table 1: Recommendations on the food-producing animal populations and samples to collect for the MRSA monitoring

Animal populations	MRSA	
	Where to collect	Samples to collect
Monitoring recommended to be performed consistently on a regular basis (every third year)		
Broilers	Farm	Boot swab[(a)]
Fattening pigs	Slaughterhouse/Farm	Pool of nostril swabs[(b)]/Boot swab[(c)]
Dairy cattle	Dairy farm	Bulk tank milk
Monitoring recommended to be performed consistently on a regular basis, if production exceeds 10 million tonnes slaughtered/year (every third year)		
Fattening veal calves (under 1 year of age)[(e)]	Slaughterhouse	Nostril swabs
Fattening turkeys	Farm	Boot swabs[(a)]
Monitoring recommended to be performed on a voluntary basis (every third year)		
Breeders of pigs	Farm	Nose swab
Breeders of *Gallus gallus*, meat sector	Farm	Boot swab/Nose skin swab[(a)(d)]
Breeders turkeys	Farm	Boot swab/Nose skin swab[(a)(d)]
Beef animals	Slaughterhouse	Nostril swabs
Horses	Slaughterhouse	Nostril swabs

(a) In the framework of the *Salmonella* National Control Programmes, an additional boot swab sample may be obtained for MRSA testing.

(b) Sampling on farm is preferred for the purpose of assessing the risk factors for MRSA infection. In this case, larger pools of nose swabs can be collected.

(c) Sampling at slaughter or on farm depending on the considerations developed in section 4.1.1.1.

(d) Nose skin swabs have been reported to be more sensitive than boot swabs in poultry (P. Butaye, Veterinary and Agrochemical Research Center, Belgium personal communication, 2012).

(e) In certain MSs, the calf population to be monitored for MRSA may also comprise fattening veal calves older than 1 year.

Table 2: Recommendations on the food categories and samples to collect for the MRSA monitoring

Food	MRSA
	Where to collect
Monitoring recommended to be performed on a voluntary basis (every third year)	
Fresh broiler meat	Cutting plant or at retail
Fresh turkey meat	Cutting plant or at retail
Fresh pork	Cutting plant or at retail
Fresh beef	Cutting plant or at retail
Fresh veal	Cutting plant or at retail
Raw milk and/or raw milk products	Dairy/processing plant or at retail

4. Recommendations on the methodologies considered most relevant for MRSA monitoring from a public health perspective

4.1. Sampling designs

Some recommendations are given below for sampling in MRSA monitoring programmes of several food-producing animal species and production systems, as well as of some foodstuffs of animal origin (meat and raw milk). The objectives of the programme, the sensitivity of detection, the cost-effectiveness of the approach and the ease of implementation were considered in order to formulate these recommendations.

4.1.1. Samples for MRSA monitoring in food-producing animal populations

4.1.1.1. Samples for monitoring MRSA in swine

Sampling of pigs to determine the prevalence of MRSA is crucial for several reasons, including assessment of the occupational exposure of people working with live pigs, such as farm workers, veterinarians and slaughterhouse personnel. In many MSs, the prevalence of MRSA in pigs (or in pig holdings) is assumed to be high. Monitoring of MRSA in pigs is important, and should be performed consistently and on a regular basis in all MSs. It is proposed that each MS should monitor MRSA in pigs at the slaughterhouse or on farm. The choice should be based on the considerations detailed below.

Sampling pigs at the slaughterhouse requires, in general, a smaller sample size than farm-level sampling, as the highest prevalence is expected at this stage of the production chain, because of the potential for cross-contamination during transport and lairage. In particular, in the case of MSs that have little information on MRSA occurrence in their pig population or which have a very low prevalence of MRSA in their pig population, slaughterhouse sampling may provide an easy and effective means of determining the presence of the bacterium in the pig population. In addition, monitoring the diversity of MRSA strains is usually more effectively done at the slaughterhouse, as a higher diversity of MRSA subtypes is more likely at this stage of the production chain than on the farm, which is of interest for comparison with the MRSA strains prevalent in humans.

Monitoring of MRSA at the slaughterhouse through nasal swabs is preferred mainly for practical reasons related to the feasibility, the cost-effectiveness and the sensitivity of the sampling. On any given day of operation, five nasal swabs (from five different pigs) can be taken and pooled into one sample for further testing. It is believed that a pool size of five offers a good compromise between sensitivity and the amount of effort required.

MRSA contamination of pigs can occur in slaughterhouses and clustering is expected between animals depending on the level of contact between animals during rearing, transport, housing in lairage and even sequence of passing through the slaughterhouse. To maintain independence between samples, minimum physical contact of sampled batches is important, before sampling and ideally only one pooled sample per slaughterhouse should be obtained on any one day. Nevertheless, to improve the feasibility of the sampling and in certain MSs, limit the number of sampling visits to the slaughterhouses, the sampler should ensure that sampled animals are slaughtered at a minimum time interval to avoid direct cross-contamination.

Sampling of pigs on farm is advisable for countries where MRSA prevalence in pigs is important and which wish to estimate this prevalence at the farm level and/or assess better the epidemiology of MRSA. In this case, it is suggested that 5 pools of 5 nasal swabs from each sampled farm are obtained and examined. If this practice is deemed economically unfeasible, then, alternatively, boot swabs can be obtained for MRSA testing (one swab from a pair of boots per farm). However, it should be noted that the sensitivity of detection of this practice will probably be lower. In all cases, it is proposed that on-farm MRSA monitoring should focus on fattening pigs during the last 2 months of the fattening period.

Breeding pigs can also be sampled, but this is considered of lower priority, and therefore is not proposed as part of routine monitoring. When monitoring MRSA in breeding pigs, samples can still be in the form of nasal swabs, as described above for fattening pigs.

4.1.1.2. Samples for monitoring MRSA in cattle

For MRSA screening and monitoring in dairy cattle, sampling of bulk tank milk in dairy farms is proposed, as bulk tank milk contains on the one hand bacteria that have been present in the milk, but may also harbour environmental bacteria. In an MRSA monitoring programme, it is recommended that 25 mL of bulk tank milk be sampled at the farm. As *S. aureus* is an important mastitis pathogen (Vanderhaeghen et al., 2010; Spohr et al., 2011), it is most likely to occur in milk from mastitic cows, and bulk tank milk always tested positive in studies on MRSA as a cause of mastitis in dairy cows (Vanderhaeghen et al., 2010; Spohr et al., 2011). Furthermore, it should be emphasised that collection of milk samples at the dairy plant may not be optimal because of the risk of cross-contamination of the samples occurring during automated collection by the technical equipment of the lorry.

In veal calves (under 1 year of age), sampling of animals has proven to be more sensitive than environmental sampling. It is proposed to monitor MRSA in veal calves through nasal swabs at the slaughterhouse. A sampling approach based on pools of five nasal swabs can be used in the same way as recommended for the sampling of pigs at slaughterhouse. Naturally, the same considerations as for slaughter pigs, concerning possible clustering, are also relevant for the sampling of veal calves at the slaughterhouse. However, routine MRSA monitoring in veal calves is suggested only for those MSs that have an important veal calf production, for example exceeding 10 million tonnes slaughtered/year. Beef animals can also be sampled in the same way, at the slaughterhouse; however, it is suggested that monitoring of these animals is performed on a voluntary basis.

4.1.1.3. Samples for monitoring MRSA in poultry

Regulation (EC) No 2160/2003[9] foresees mandatory *Salmonella* control programmes in flocks of *Gallus gallus* as well as of turkeys. Sampling on poultry farm is therefore feasible, as it may be conducted in conjunction with that of national *Salmonella* control programmes. Representative samples of broiler flocks and fattening turkey flocks subjected to official sampling in the course of *Salmonella* control programmes should also be tested for MRSA. As handling animals causes stress and, under unfavourable circumstances, may even be associated with losses, environmental sampling should be considered as an option for MRSA monitoring purposes despite its potential limitations with respect to sensitivity. Both boot swab and pooled dust samples have been used as environmental sample on farm to investigate for MRSA. A study in turkey flocks reported no sensitivity issue with pooled dust samples (Richter et al., 2012) similar to those observed in herds of pigs (Broens et al., 2011c). However, the sample size of that study was limited. It is considered that boot swab sampling can be better standardized in different poultry farms, mainly because both official samplers and farm personnel are familiar with the procedure. Therefore, it is suggested that preference is given to boot swabs, stressing, however, that an additional pair of boot swabs should be taken per flock from those already obtained for the *Salmonella* control programmes, since the laboratory testing procedures are very different for *Salmonella* and MRSA. In the case of fattening turkeys, it is proposed that routine monitoring be implemented only in MSs which have a substantial turkey production (for example exceeding 10 million tonnes slaughtered/year).

4.1.2. Samples for monitoring MRSA in foodstuffs

Monitoring of MRSA in food can be done with different methodologies and at different stages of the food chain, depending on the defined objectives of the monitoring programme, the MS-specific production details and MRSA status. In any case, it is proposed that such monitoring is done on a voluntary basis, depending on MSs' specific needs.

[9] Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents. OJ L 325, 12.12.2003, p. 1–15.

Considering the potential for consumers' exposure, MSs may carry out sampling of (fresh) meat; however, it is recommended this is done on a voluntary basis. Samples can be collected either at the cutting plant or at retail level. Sampling at retail is more relevant to the assessment of potential for consumers' exposure and, therefore, it is recommended over sampling at previous stages in the food chain. On the other hand, it is more complex and laborious than sampling carcases at the slaughterhouse or sampling fresh meat during further processing at cutting plant. Moreover, sampling at retail presupposes that the origin of the meat is reliably identified as part of the monitoring procedure. Domestic meat sampling should be prioritized, as it can also yield, indirectly, some useful additional information on the infection status of the production sector in the MS and of the contamination status of the slaughter line. In any case, monitoring at the cutting plant and at retail should be designed and reported separately, as they may refer to different source populations (e.g. domestic vs. imported production, etc.).

Sampling at processing more easily enables to target meat of domestic production, but it may exclude imported meat and may need to be supported by sampling of imports from third countries at entry into the EU. Several studies have revealed substantial differences in contamination rates of domestic and imported meat with respect to MRSA (de Boer et al., 2009). Moreover, there is a considerable variability among MSs with respect to the prevalent subtypes of MRSA in meat, and also between third countries and the EU. In the case of sampling at retail, products from domestic and imported raw material should be differentiated. Regardless of the place of sampling, a stratified sampling plan is proposed.

As previously mentioned, raw milk and raw milk products may contain MRSA. However, when using bulk tank milk for sampling dairy herds, these production chains are covered at the most likely source of contamination. Since milk is usually heat treated before consumption or use for the manufacture of milk products, the sampling of raw milk is primarily used for the monitoring of MRSA occurrence in dairy farms. This monitoring can be done as described above for monitoring MRSA in dairy cattle. However, if the raw milk is likely to be consumed or used for manufacturing of milk products without prior heat treatment, monitoring of raw milk and products made from raw milk may also be relevant in order to assess consumer exposure. In this case, monitoring at the dairy plant or at retail may be preferable, and samples of 25 mL of raw milk and 25 g of products thereof should be obtained.

4.1.3. Sampling frequency and targeted monitoring

Considering the current epidemiological situation and related scientific knowledge, no mitigation measures that might be able to modify the prevalence are expected to be implemented in the near future. Since the prevalence of MRSA among these animal populations is not expected to change rapidly over a 1- to 2-year period, monitoring every 3 years should provide the necessary information on the situation in each particular country and at the EU level. It is therefore proposed that all MSs carry out this monitoring at 3-year intervals, covering the different animal populations targeted on a rotating basis. It would also be desirable that all MSs conduct the monitoring in the same animal population in the same year. Repeated monitoring over time will enable the following of trends in the prevalence of the different MRSA subtypes in monitored animal populations in the EU and individual MSs.

4.1.4. Sampling plans

- **General considerations on a representative and random sampling design**

From a sample examined, inferences will be made about parameters in the entire population from which the sample originates. For this reason, formal randomised sampling strategies should ideally be implemented, allowing for proper statistical data analysis and reducing the effect of sampling bias. Random sampling in each targeted animal population ensures the representativeness of the samples collected, makes inference to the entire population possible, and reflects the variability in managerial and hygienic practices in holdings and different country regions. Such samples are, ideally, derived from active monitoring programmes. In such cases, it is important that the bacterial isolates originate from healthy animals sampled from randomly selected holdings/flocks or randomly selected within the

slaughterhouses. An approximately equal distribution of the collected samples over the year enables the different seasons to be covered. In addition, when the sampled population (e.g. batches) is structured at several levels, then the sampling plan (and the subsequent statistical analysis) should account for this structure. If diseased animals are sampled, for example dairy cows with clinical mastitis, the corresponding results should be reported separately. In this case, the sample may not be representative of the entire population; therefore the results should be interpreted with caution.

- **Sampling plan of MRSA in poultry populations monitored on farm**

For the monitoring of MRSA in poultry populations, an unbiased estimate of the prevalence, diversity and proportion of resistance may be obtained through a sampling frame covering all epidemiological units (flocks) of the national production, such as that of the national control programmes for *Salmonella*. The epidemiological unit for the various poultry populations concerned is the flock, because most holdings practise all-in–all-out production.

- **Sampling plan of MRSA in animal populations monitored at the slaughterhouse**

In the case of fattening pigs and fattening veal calves (under 1 year of age), it is proposed that MRSA monitoring is primarily based on the collection of samples at the slaughterhouse. Sampling performed at the slaughterhouse is preferred as in many MSs it will be the most cost-effective way to collect samples. It is proposed that at least 60-80 % of the domestic animal population in a MS is included in the sampling frame, which means that slaughterhouses processing at least 60-80 % of the domestic animals (starting with the slaughterhouses with the largest throughput) are eligible for sampling. In the case of sampling performed at the slaughterhouse, an active monitoring programme should be based on random sampling of healthy animal carcasses (e.g. calves, pigs). The sampling plan should preferably be stratified by slaughterhouse by allocating the number of (pooled) samples collected from each slaughterhouse in proportion to the annual throughput of the slaughterhouse. An approximately uniform distribution of the collected samples over the year enables the different seasons to be covered. Only one representative pool of five nasal swabs per epidemiological unit (e.g. farm), in each case derived from five carcasses, is gathered to account for clustering.

4.1.5. Sample size

- **Sample size for the purpose of monitoring MRSA prevalence**

The target sample size may vary depending on whether the sample size is calculated for the purpose of assessing prevalence or for the purpose of determining a trend. The sample size is function of the prevalence expected (i.e. the proportion of samples expected to test positive), and the initial prevalence when one aims at detecting changes in the prevalence (i.e. a trend). Moreover, the sample size may also differ greatly depending on the accuracy of the prevalence estimate, the magnitude of the change that it is hoped will be detected and the sensitivity and specificity of the test. In addition, for a number of MSs, the population of eligible carcasses/flocks may be of a limited size (for example, breeding flocks of poultry), which may induce a smaller sample size. Conversely, MSs may wish to collect more samples, for example to compensate for possible clustering.

As the prevalence of MRSA among the targeted food-producing animal populations differs widely between MSs, it is acknowledged that the sample size needs to be adapted to the local epidemiological situation and calculated at the MS level. For example, to estimate the prevalence of MRSA infection among a particular population of batches of fattening pigs at slaughter, for which a prior prevalence of 12 % of positive batches is expected, a minimum of 254 eligible batches should be sampled at random from MS slaughter pig populations. This sample size provides an absolute accuracy of $\pm$ 4 % for a prevalence estimate of 12 %. In addition, if the aim is to monitor changes in the proportion of colonised animals over time, a detectable difference may be calculated based on the sample size recommended for estimating prevalence.

- **Sample size for the purpose of monitoring the antimicrobial resistance of MRSA**

As previously reported (EFSA, 2012a), it is acknowledged as desirable, from a public health perspective, that an adequate target number of 170 MRSA isolates is susceptibility tested per study animal population, per country and per year. The number of biological samples that needs to be collected from each animal population in order to achieve 170 MRSA isolates depends on the prevalence of MRSA. In the particular case of very low MRSA prevalence, whenever a large number of samples needs to be collected to achieve a sufficient number of isolates, a practical solution is the collection of a minimum number of randomised samples (enabling to conclude that the prevalence is likely not above a certain level if no positive are detected: see below) and/or implement a passive surveillance scheme based on strains isolated from clinical cases deriving from targeted or systematic sampling.

- **Sample size for the purpose of investigating 'absence' of MRSA**

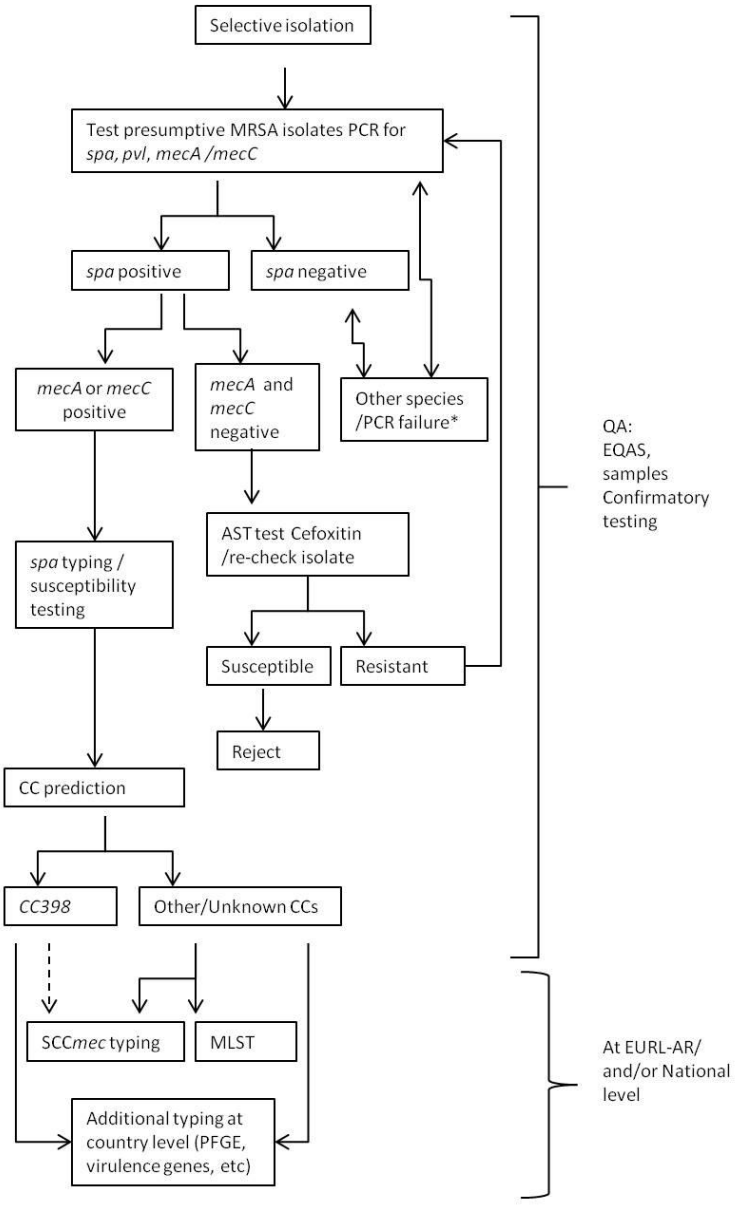
In MSs in which MRSA has not been found, surveys may be designed with the purpose of confirming MRSA prevalence is likely not above a certain level. Sample size for such surveys can be calculated using freely available software as, for example, FreeCalc[10]. These calculations need to take into consideration the sensitivity and specificity of the diagnostic test that is used and the 'design prevalence' that is the minimum prevalence that the survey is designed to detect. The relevant calculations are detailed in Cameron and Baldock (1998). For example, using FreeCalc, if one assumes a test with 90 % sensitivity, 100 % specificity, a minimal expected prevalence of 5 %, a desired probability of type I and type II error of 5 % and a very large population of animals/batches for which inferences are sought, the required sample size would be 66 animals. The same calculation for a minimal expected prevalence of 2 %/3 % yields a required sample size of 165/110 animals, while if the sensitivity of the test was 80 % rather than 90 %, then the required sample size would be 186 animals (for a minimal expected prevalence: 2 %).

10 FreeCalc – Cameron, AR. Software for the calculation of sample size and analysis of surveys to demonstrate freedom from disease. Available for free download from http://www.ausvet.com.au

4.2. Analytical methods in routine monitoring of MRSA and quality control

4.2.1. Isolation, identification and typing of MRSA

In order to detect MRSA in a given sample, several successive analytical steps are needed. The first step is to isolate presumptive MRSA. Confirmation of MRSA is then obtained by the presence of *mecA* or *mecC* by multiplex Polymerase Chain Reaction (PCR) or, in isolates negative for these genes, by phenotypical test for resistance to cefoxitin. MRSA confirmed isolates are further *spa*-typed in order to determine the corresponding CC. Isolates for which no CC can be inferred from the *spa*-type should be further typed by MLST-typing. Complementary analytical tests can also be performed to further characterise isolates. The different analytical steps and corresponding methods are presented in Figure 1. The choice and number of isolates depends on the purpose, for example characterisation of epidemiologically related isolates, research or verification of novel findings.



*Multiplex PCR does not contain internal control, beware that in case no bands are observed the PCR might need to be repeated with new DNA samples and or test repeated using another PCR method.
Dotted arrow- A sample of the CC398 isolates will be selected for SCC*mec* typing.

Figure 1: Flowchart for isolation, identification and typing of MRSA.

Isolation of MRSA differs depending on sample type and animal population. In order to ensure that the prevalence obtained in an animal population can be compared between years and different countries, it is important to harmonise the isolation procedure. Therefore, detailed protocols of the isolation procedure, including the preparation of samples, for different sample types and animal populations as well as in food will be made available on the European Union Reference Laboratory for AMR (EURL-AMR) homepage and the EURL-AMR can be contacted for technical support.

4.2.1.1. Isolation of MRSA and identification

The isolation method recommended[11] by the EURL-AMR for testing MRSA in food-producing animals and food samples requires a pre-enrichment step and a selective enrichment step, regardless of sample type.

- The pre-enrichment is in Mueller-Hinton broth containing 6.5 % sodium chloride (NaCl) incubated for 18-24 hours at 37°C, which selects for staphylococci and other salt-tolerant bacteria (Agersø et al., 2012).
- Following the pre-enrichment step, a selective enrichment is performed in Tryptone Soya Broth (TSB) containing 4 mg/L (or 3.5 mg/L) cefoxitin and 75 mg/L aztreonam incubated for a further 18-24 hours at 37°C. This step selects for MRSA and other resistant bacteria such as coagulase-negative staphylococci (CNS) and resistant enterococci.
- To obtain presumptive positive colonies of MRSA, plating on a chromogenic MRSA-selective and indicative agar is performed. The plate recommended by the EURL-AMR is the Brilliance MRSA screen 2 agar (Oxoid)[12] incubated for 22-26 hours at 37°C. As the screen plate may not always be 100 % indicative and selective, it is recommended that presumptive MRSA colonies are subcultured on blood plates for 24-48 hours at 37°C to look for characteristic morphology and haemolysis, and to perform catalase testing on doubtful isolates.

The screen plates available may produce to some extent false positive results, so a number of presumptive isolates need to be subcultured and confirmed by molecular testing of MRSA.

4.2.1.2. Confirmatory testing for *S. aureus* and MRSA using multiplex PCR

Molecular testing needs to be performed on the presumptive positive MRSA isolates to confirm methicillin resistance (gold standard). To limit the amount of work required, initially only one of the presumptive isolates should be tested and the remaining tested sequentially until one has been identified as MRSA. In this view, it is necessary to perform *mecA* gene detection using simple or multiplex PCR amplification methods. It is recommended that a reliable PCR method be used and that the procedures be harmonised.

For this purpose, it is recommended that a multiplex PCR be used that allows, in the same PCR:

- species identification of *S. aureus* with detection of the staphylococcal protein A gene (*spa*);
- detection of the methicillin resistance determinant, including both *mecA* and the recently described *mecC*; and

[11] Several methods are reported in the literature, and they vary to some extent. Some authors use a combined salt-antimicrobial enrichment broth with a lower salt concentration and antibiotics in one step, but this medium has been evaluated only on human samples (Böcher et al., 2010). Others use a selective enrichment step with Phenol Red-mannitol broth instead of Tryptone Soya Broth (TSB). Others again have found incubation for 48 hours to be useful for pre-enrichment or prefer to skip the selective enrichment step entirely. In general, it is believed that the method can be optimised, e.g. by lowering the antibiotic (aztreonam) concentration in the selective enrichment step, but supportive data are lacking.

[12] The screen plate recommended by the EURL-AMR is more indicative than the previously available plate manufactured by Oxoid (MRSA screen agar), but other commercial chromogenic agar plates are also available. Alternatives such as MRSA-Screen (Oxoid), MRSA Select (Bio-Rad) and MRSA ID (bioMérieux) have been evaluated in scientific studies. These plates vary in selectivity and are to some extent dependent on the sample matrix (Graveland et al., 2009; Verkade et al., 2011).

- simultaneous detection of the PVL gene.

This multiplex PCR has recently been described by Stegger et al. (2012) and is currently recommended by the EURL-AMR, as it has been shown to be easy to set up and provides reliable results for *mecA*, *mecC*, *pvl* and *spa* after amplification. In addition, the PCR product can be further used for sequencing of the *spa* amplicon directly, providing also the basis for *spa* typing. However, the PCR does not contain an internal control and, therefore, negative results need to be confirmed. If an isolate is "truly" negative by PCR, MRSA needs to be confirmed by phenotypical testing for cefoxitin (by disc diffusion or E-test).

PVL-positive isolates (or a subset if several isolates of the same *spa*-type are found) should be confirmed by Deoxyribonucleic Acid (DNA) sequencing or further testing for PVL subtypes: the National Reference Laboratories (NRLs) or the EURL on staphylococci can support the verification. The EURL-AMR can also provide support on the verification and provide information on the novelty of the finding in a given species or *spa*-type.

Furthermore, this multiplex PCR method has been introduced successfully to the NRLs on AMR that participated in the molecular characterisation training course organised by the EURL-AMR in November 2011, where the method was carried out by all participants in the laboratory. The EURL-AMR can provide protocols, positive controls and technical assistance to help laboratories involved in MRSA monitoring to set up the methods.

Nevertheless, if further information is needed, additional species markers, such as *nuc*, may be tested separately at MS.

Isolates verified as MRSA should be saved and stored at -80°C at least for 5 years. This is to allow, for instance, later testing for antimicrobial susceptibility or other types of characterisation if requested by the EC or for research or other purposes at a national level. Isolates sent to the EURL-AMR will also be stored for a minimum of 5 years. Isolates should be stored under conditions not allowing changes in their properties (-80°C). If the laboratory in charge does not have the available storage capability, isolates could be forwarded to the EURL-AMR, which can store these isolates.

4.2.1.3. Determination of *spa*-types, sequence types and clonal complexes

In the framework of the monitoring of MRSA, positive MRSA isolates should be subtyped consistently to assess the genetic diversity of the MRSA prevalent in animal populations and food so that possible clonal spread within and between different animal populations/food categories can be identified (phylogenetic analysis) and comparisons between animal and human MRSA can be performed, thus enabling a potential link with MRSA strains of human origin (www.seqnet.org) to be established. For this purpose, the *spa*-type, the sequence type (ST) and the clonal complex of MRSA isolates should be determined. Detailed definitions of *spa*-typing, ST and CC as well as of the related typing methods are given in the glossary.

- ***Spa*-typing**

For the monitoring of MRSA, s*pa*-typing should be performed in all isolates identified as MRSA using a standard method. This method is simple and highly reproducible between laboratories and it gives a good indication of the population structure, although it does not have very high discriminatory power. Identification of the *spa*-type may allow clustering of related types and also the assignment into CCs. In general, isolates with a similar succession of *spa* sequences belong to closely related STs, which can be assigned to the same CC. Typically, direct assessment of the ST is carried out by comparing the *spa*-types with databases of known and typed strains. Information derived from databases is also used to assign isolates directly to known CCs. However, it has been reported that one *spa*-type might actually be found in strains with different background, such as is the case for t899, which can be found among strains belonging to CC398 and CC9. MLST enables confirmation of concordance between *spa*-typing and allocation of a given isolate to a specific ST/CC.

Amplification of the *spa* gene may be performed using a simple PCR method as described by Shopsin et al. (1999), or directly from the multiplex PCR reaction described by Stegger et al. (2012), recommended above for the identification of MRSA. Sequencing may be carried out by the individual laboratories or outsourced to sequencing services; the results can be analysed using the Ridom software or using a *spa*-typing Plugin in Bionumerics which allows determination of the *spa*-type by comparing the data with the *spa*-types at the ridom *spa* server (http://spaserver2.ridom.de/) and, therefore, assigning the corresponding *spa*-types.

The NRLs and other laboratories belonging to the EURL-AMR network were trained in *spa*-typing at training courses provided in 2009 and 2011 by the EURL-AMR, and *spa*-typing has been an optional module of the MRSA External Quality Assessment Scheme (EQAS) run by the EURL-AMR since 2009. If laboratories do not have the capacity to perform *spa*-typing or sequence analysis, they may ask for the assistance of the EURL-AMR or another laboratory. The EURL-AMR can provide support on the interpretation of results on novel combinations of *spa*-types and animal species. Novel combinations should always be confirmed by MLST, and support for this may be provided by the NRL or EURL-AMR.

- **Multilocus sequence type and clonal complex assignments**

For the routine monitoring of MRSA in the MSs, it is recommended that MLST (see Glossary) be performed on a convenient subset of isolates of different *spa*-types. MLST enables the assignment of a ST to each MRSA isolate, which can be subsequently allocated to specific CCs. At least one isolate belonging to any novel *spa*-type identified at the MS level, as well as representatives of the major groups of s*pa*-types found, in particular those not belonging to CC398, should undergo MLST to confirm concordance between *spa*-typing and allocation of a given isolate to a specific ST/CC. This should allow an overview of the population structure of the MRSA detected from the diverse sources investigated in the EU. MLST is also recommended if a CC type cannot be inferred from the *spa*-type.

Standard procedures and submission of sequence data to MLST assignment tools can be found at the MLST server (www.mlst.net). PCR amplification and sequence analysis for MLST in *S. aureus* has also been included in the courses provided to the EURL-AMR network both in 2009 and 2011. Furthermore, population structures can be drawn using the e-Burst algorithm (http://eburst.mlst.net).

4.2.1.4. Complementary typing tests for epidemiological purposes

In certain situations, for further epidemiological investigations or in research, it may be desirable and useful to perform additional genetic analyses of certain MRSA isolates, in particular through SCC*mec* typing, DNA analysis by pulsed-field gel electrophoresis (PFGE) or whole-genome sequencing (WGS). Detailed definitions of these typing methods are given in the Glossary. Data on the main characteristics, interest and limitations of these typing methods, supplementary to those given hereafter, are also presented in the Appendix.

Typing of the SCC*mec* cassette (see Glossary) is useful for achieving an insight into these elements and their variants in the MRSA population, which might enable the acquisition events leading to emergence of MRSA strains from MSSA clones to be estimated. It is advisable to perform SCC*mec* typing on a convenient subset of MRSA isolates only, including a representative sample of CC398 strains and possibly all non-CC398 strains. In the monitoring of LA-MRSA, it is expected to find a majority of SCC*mec* types IV and V, but others may also be detected (see Appendix).

In the experience of the EURL-AMR, the Kondo method (Kondo et al., 2007) is adequate for basic typing (multiplex 1 and 2) and for the sub-typing of type IV SCC*mec* elements. However, additional testing might be necessary for a proportion of the isolates carrying *mecA*. For any isolate carrying *mecC*, the method applied would be the specific method focused on SCC*mec* XI (García-Álvarez et al., 2011). Although the execution of SCC*mec* typing using the Kondo method has already been introduced to the NRLs on AMR through the training courses held by the EURL-AMR in 2009 and 2011, the use of this typing method may cause technical difficulties in the set-up and standardization

of the PCR methods as well as in interpreting the results at country level. Therefore, it is presently recommended that SCC*mec* typing be performed centrally by the EURL-AMR or by other laboratories with the necessary skills and capabilities.

The analysis of chromosomal DNA by PFGE (see Glossary and the Appendix) has been used to reveal clonal structure and relationship between MRSA isolates and as well between *S. aureus* isolates in general. PFGE is therefore considered a useful technique to investigate and characterise outbreaks. This technique has been extensively used to provide a comprehension of the epidemiology of MRSA strains in both animals and humans.

The WGS of a sample of MRSA isolates may also be considered in the future, as it provides the opportunity to obtain additional information, and it may even replace other typing schemes, such as MLST, antimicrobial susceptibility testing and virulence gene investigation, among others.

Commercial microarray tests are available (Monecke et al., 2008), which can be used to investigate MRSA isolates for a range of virulence and other genes, for example staphylococcal enterotoxin genes. The results of such investigations for a number of virulence genes can be used to sub-classify and type strains for which other phenotypic (for example antimicrobial resistance pattern) and genotypic (for example *spa*-type) data are available. Such investigation complements the use of techniques such as PFGE and may be used to track the stability or ongoing evolution of MRSA strains, as well as for comparative purposes, when examining possible relationships between isolates from food, animals and man. However, certain virulence characteristics may be borne on plasmids and may therefore not be entirely stable.

4.2.1.5. Quality control for identification and typing of MRSA

A quality assurance element should be included in any MRSA monitoring programme to detect any potential differences between laboratories carrying out detection and susceptibility testing of MRSA, particularly if laboratories other than the NRLs on AMR are involved. At the EU level, proficiency tests are organised by the EURL-AMR in identification and typing, as well as susceptibility testing of staphylococci. This is performed annually on a revolving basis for the NRLs on AMR, supporting the harmonisation process.

In addition, it is recommended that MRSA isolates are stored at the competent NRLs at a temperature of -80°C, to ensure viability and avoid changes in strain properties, for a minimum period of 5 years. If necessary, or in case of doubt, or for the purpose of quality control, a convenient subsample of confirmed and non-confirmed MRSA isolates recovered from the routine monitoring programme may be sent for re-testing to the EURL-AMR to assess standardisation of identification and typing. Isolates sent to the EURL-AMR may be stored for any further testing and/or confirmation, if needed.

4.2.1.6. Phylogenetic analysis of the relationship between *spa*-types isolated

The *spa*-typing results may be used to infer the CCs from the Ridom *spa*-server, which records previously described *spa*-type/CC relationships. Conversely, in the case of new *spa*-types, the ST needs to be determined (either by standard PCR and sequencing or from WGS data) to allocate the CCs. Moreover, *spa*-typing results can be used to cluster *spa*-types to obtain information on their relationship, by using available tools, such as the minimal spanning tree, which can be obtained by analysing the typing results using, for example, the corresponding algorithm in Bionumerics software (Applied Maths, St-Martens-Latem, Belgium), and/or the Based upon repeat (BURP) algorithm available in the Ridom StaphTypeTM (Ridom GmbH, Würzburg, Germany) software. Algorithms calculate population modelling networks in cluster analysis and create phylogeny trees based on *spa*-repeats, which serve as an initial grouping of the isolates of closely related *spa*-types within certain related *spa*-groups. By the phylogenetic analysis, isolated strains can be organised into CCs of closely related strains that share alleles in common. The result of the MLST analysis may be also compared with the *spa*-groups in order to confirm the correct distribution into specific CCs.

4.2.2. Technique for antimicrobial susceptibility testing of MRSA

Regarding the antimicrobial susceptibility testing of MRSA, the recommendations on the analytical methods in routine monitoring and on quality control previously made for the AMR monitoring and presented in the EFSA Scientific report on technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food (EFSA, 2012a), should fully apply. These recommendations rely on the phenotypic monitoring of the resistance to a common set of antimicrobial substances, based on standardised dilution methods with appropriate dilution ranges and ECOFFs as interpretative criteria for resistance. For MRSA, the corresponding technical specifications for the common set of antimicrobials, dilution ranges and ECOFFs are given in the next section of this report.

5. Recommendations on antimicrobials, epidemiological cut-off values and optimum concentration ranges to be used for susceptibility testing of MRSA isolates

5.1. Harmonised panel of antimicrobials for susceptibility testing of MRSA

AMR monitoring through antimicrobial susceptibility testing of MRSA isolates is recommended. No guideline for antimicrobial susceptibility testing of *S. aureus* has yet been published by the EFSA. However, in the framework of the EU-wide baseline survey on MRSA in breeding pigs (EFSA, 2009b), it was proposed that the microdilution method be used to test the susceptibility of MRSA isolates to at least the following antimicrobial agents: ciprofloxacin, erythromycin, fusidic acid, gentamicin, linezolid, mupirocin, sulfametoxazole/trimethoprim, tetracycline, chloramphenicol, vancomycin and quinupristin/dalfopristin. To provide continuity of monitoring data and allow epidemiological tracing of isolates with particular patterns of resistance, it is recommended that these antimicrobials should continue to be included in future protocols. Testing susceptibility to additional antimicrobials is also considered useful in some circumstances, but a cost-effective approach does not currently allow the inclusion of complementary antimicrobials within the harmonised set of substances for which testing is recommended. Indeed, inclusion of these antimicrobials on primary plates is likely to be at the expense of other antimicrobials.

This rationale provides the information used to assess and discuss the inclusion of antimicrobials in the recommended monitoring scheme for MRSA. Antimicrobial agents were included if they were considered (1) relevant for human therapeutic treatment of *S. aureus* infections, (2) relevant to detect new resistance mechanisms of public health importance that may spread to other important human pathogens and (3) relevant if the antimicrobial agent is used for veterinary therapy and may select for specific multi-resistant phenotypes of importance for therapeutic treatment of human infections.

The proposed lists of antimicrobials to be included in AMR monitoring in MRSA are the following:

- Recommended set: cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, linezolid, mupirocin, quinupristin/dalfopristin, sulfamethoxazole/trimethoprim, tetracycline, tiamulin, vancomycin.
- Optional set: ceftobiprole, kanamycin, tigecycline, fusidic acid, daptomycin.

In light of the experience gained through consistent monitoring of susceptibility testing of MRSA, regular review, future developments and refinement of technical specifications are expected in the future, particularly regarding the harmonised antimicrobial panels, ranges of concentration and ECOFFs.

5.1.1. Antimicrobials to be inserted in the recommended panel of antimicrobials

Cefoxitin: Cefoxitin is a semi-synthetic cephamycin (i.e. the second generation of cephalosporins). It is considered by the World Health Organisation (WHO) as a highly important antimicrobial for human medicine as it is indicated in severe infections in children. Furthermore, cefoxitin is an essential marker for phenotypic detection of methicillin resistance in *S. aureus* due to the *mec* genes (*mecA* and *mecC*), which confer resistance to all beta-lactam drugs by target replacement. In the framework of the monitoring of MRSA, testing of cefoxitin gives an additional confirmation of the MRSA status of the *S. aureus* isolates selected.

Chloramphenicol[13]: Chloramphenicol is a phenicol drug which was previously used in animal production, but was banned at the start of the 1990's owing to toxicity issues. It is, however, related to the fluorinated phenicols, such as florfenicol, which is currently used in production animals. Its use in humans is usually limited to topical treatment. However, it is sometimes used in some geographical areas, where the availability of other therapies is limited, for the treatment of bacterial meningitis,

[13] Testing for either florfenicol or chloramphenicol has been considered. Owing to availability of data from human medicine, chloramphenicol is preferred.

typhoid and non-typhoid fever and respiratory infections and, therefore, is classified by WHO as a highly important antimicrobial. Cross-resistance may occur with florfenicol, which is used for treatment of production animals. In *S. aureus*, resistance is mainly due to the plasmid-borne chloramphenicol acetylase of type A (*catA*), which encodes resistance to chloramphenicol but not to florfenicol. However, two resistance genes mediate both chloramphenicol and florfenicol resistance, *cfr* (rRNA methylation) and *fexA* (active efflux). The *cfr* gene is of grave concern, as it has been detected among MRSA isolates of animal origin and is a plasmid-mediated gene encoding for a 23S rRNA methyltransferase conferring resistance to all phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics.

Ciprofloxacin: Ciprofloxacin is a member of the fluoroquinolone group of antimicrobials. Ciprofloxacin, among other fluoroquinolones, is widely used in human medicine whereas enrofloxacin is used in veterinary medicine. In comparison with ciprofloxacin, enrofloxacin is slightly less potent, but complete cross-resistance occurs between the two substances. Fluoroquinolones are among the top three critically important antimicrobials classified by WHO. Resistance to fluoroquinolones in *S. aureus* is caused by point mutations in the topoisomerase genes *grlA*, *grlB* and *gyrA and gyrB*, which can arise as single or multiple mutations conferring increases in the Minimum Inhibitory Concentrations (MICs) of quinolones. Therefore, resistance to fluoroquinolones spreads vertically and resistance to these drugs can be used as marker for certain clones.

Clindamycin: Clindamycin is a lincosamide antibiotic. It is usually used to treat infections with anaerobic bacteria but can also be used to treat some protozoal diseases, such as malaria. It is also very commonly used for the topical treatment of human skin infections caused by, for example, staphylococci, and it is used to treat infections caused by MRSA. Resistance has been observed in *S. aureus*, including MRSA, and is often caused by *erm* genes, which can cause both constitutive and inducible resistance. Furthermore, resistance to lincosamides and pleuromutilins is caused by active efflux due to the presence of *vga*(A) variants, *vga*(C) and *vga*(E), which have been reported in MRSA. Moreover, resistance conferred by *cfr* genes, which cause resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics, has also been found.

Erythromycin: Erythromycin and other macrolides are widely used in both human and veterinary medicine and they are listed as "Critically Important Antimicrobial agents for treatment of human infections" by WHO. Macrolides, e.g. tylosin, are commonly used in animal production and the prevalence of resistance among MRSA CC398 is high. The mechanism of resistance is often due to *erm* genes, which, if constitutively expressed, confer cross-resistance to all macrolides as well as to lincosamides and streptogramins of the B type (MLSB resistance). Other erythromycin resistance types found in staphylococci are the efflux mechanisms encoded by the *mef* or *msr* genes.

Gentamicin: Gentamicin is an aminoglycoside antimicrobial drug related to kanamycin, amikacin, tobramycin, apramycin and netilmycin. It is used for treatment of serious infections in both humans and animals, either alone or in combination with beta-lactam drugs for a larger spectrum of activity. Gentamicin is among the drugs classified as critically important by WHO because of its broad spectrum of indications for which there are few other therapeutic alternatives. Resistance to aminoglycosides is mostly caused by enzymatic inactivation (acetylation and phosphorylation), *aacA-aph* being the main gene encoding for gentamicin resistance in *S. aureus*.

Linezolid: Linezolid is the first member of a new class of antimicrobials called oxazolidinones. It was first approved for use in 2000. It is active against most Gram-positive bacteria that cause disease, including MRSA. Linezolid inhibits protein synthesis by fixation on 23S rRNA. The mechanisms of resistance described to date are due either to mutation in domain V of 23S rRNA or to acquisition of a plasmid carrying the *cfr* gene, which also encodes for resistance to phenicols, lincosamides, pleuromutilins and A-type streptogramins.

Mupirocin: Mupirocin is used for decolonisation of MRSA in humans and is also used as a topical treatment for bacterial skin infections. In veterinary medicine, mupirocin ointment is indicated for the

topical treatment of canine bacterial infections of the skin. Resistance in *S. aureus* has been observed and categorised into low-level and high-level resistance. High-level resistant isolates often carry the plasmid-mediated *mupA* gene, whereas low-level resistance isolates have been found to carry *mupA* on the chromosome.

Quinupristin-dalfopristin: Quinopristin and dalfopristin are both members of the streptogramin class of antimicrobials. They are protein synthesis inhibitors used in combination in the proportion 30 %-70 %, respectively. They are used to treat human enterococci and staphylococci infections and confer cross-resistance to previously used growth promoters.

Sulfamethoxazole/trimethroprim: Sulfonamides and trimethoprim are used in a combination called cotrimoxazole. Both antimicrobials interfere with nucleic acid synthesis, and their combination has a remarkable synergistic effect. This combination was one of the most commonly prescribed antimicrobials before fluoroquinolones were launched in the 1980's. Its success is probably due to its broad spectrum of activity against various pathogenic bacteria, as well as some fungi and various parasites. The other side of the coin is that numerous resistance mechanisms have been described: in LA-MRSA CC398 in particular, genes *dfrA (dfrS1), dfrD, dfrG* and *dfrK* have been already detected. In order to spare wells on the panels, it was decided to test these two antimicrobials in combination. The usual ratio is 20:1 sulfonamides-trimethoprim.

Tetracycline: Tetracyclines are used in both humans and animals, including for the treatment of infections caused by staphylococci. Tetracyclines are listed as "Highly Important Antimicrobials" by WHO. Tetracycline resistance is extremely high in MRSA of animal origin (CC398) due to *tet*(M) and in some cases additional *tet*(K) and/or *tet*(L). Isolates resistant to tetracyclines are often resistant to other antimicrobial agents as well.

Tiamulin: Pleuromutilins (i.e. tiamulin and valnemulin) are mainly used in veterinary medicine, especially in swine and to a lesser extent in poultry and rabbits. A decreased susceptibility of MRSA ST398 to tiamulin has been reported recently.

Vancomycin: Vancomycin and other glycopeptides, such as teicoplanin, are used as antimicrobials of last resort to treat severe infections caused by enterococci and staphylococci in certain hospitalised patients. These antimicrobial agents are listed as "Critically Important Antimicrobials" by WHO. Vancomycin and teicoplanin are not used in veterinary medicine; however, an analogue, avoparcin, was used as a growth promoter in animal husbandry and shown to select for vancomycin resistance of the *vanA* phenotype in *E. faecium.* Use of avoparcin as growth promoter was banned in the EU in 1997[14]. Acquired resistance to glycopeptides is encoded by the *vanA* gene cluster in staphylococci.

[14] Commission Directive 97/6/EC of 30 January 1997 amending Council Directive 70/524/EEC concerning additives in feedingstuffs. OJ No L 35, 5.2.97, p.11-13.

5.1.2. Antimicrobials to be inserted in the optional panel of antimicrobials

The list below of antimicrobial substances to include in the optional panel to be tested on a voluntary basis may be complemented at the MS level based on particular epidemiological situations, animal populations monitored and specific interests. For example, when monitoring MRSA in horses, testing susceptibility to rifampicin may be of particular interest, as rifampicin may be used to treat respiratory infections due to *Rhodococcus equi* in foals. Conversely, because of its extremely limited use in animals, rifampicin might also reflect resistant bacteria originating in the human population which have colonised animals.

Ceftobiprole: Ceftobiprole is a broad-spectrum fifth-generation cephalosporin with activity against MRSA and penicillin-resistant pneumococci. It has been recently licensed in several countries (including Canada and Switzerland) for the treatment of complicated skin and skin structure infections. This new generation of beta-lactams is not used in animals, but, owing to its critical importance for human medicine, potential emergence of resistance in zoonotic bacteria should be monitored.

Kanamycin: Kanamycin is an aminoglycoside antimicrobial drug related to gentamicin, amikacin, tobramycin, apramycin and netilmycin. It is used mainly for the treatment of serious infections in humans, but to a lesser extent than other aminoglycosides. Kanamycin is among the drugs classified as highly important by WHO. In addition to resistance caused by the *aacA-aph* gene (acetylation and phosphorylation), kanamycin resistance in *S. aureus* may also be caused by the presence of other genes encoding enzymatic modifications, such as *aadA* (adenylation) and *aphA3* (phosphorylation), which have also been previously observed in MRSA of animal origin.

Fusidic acid: Fusidic acid is used in humans mainly for the topical treatment of skin and eye infections. Veterinary use is mainly for eye and skin infections in small companion animals such as dogs. The drug is primarily active against Gram-positive bacteria such as staphylococci and streptococci including MRSA. Resistance has been observed in staphylococci, and an active efflux mechanism encoded by the gene *mdeA* has been described. Resistance can also be caused by point mutations.

Tigecycline: Tigecycline is a tetracycline analogue, the first of a new class called glycylcyclines to be launched. It was approved by the Food and Drug Administration (FDA) in 2005 as an anti-MRSA drug. This broad-spectrum drug diffuses promptly from the bloodstream to tissues and has proved to be effective for the treatment of surgical wound infections, in which gut bacteria as well as MRSA can be involved. This antimicrobial is not used in animals but is considered of critical importance in human medicine. Surveillance of the occurrence of resistance in bacteria from animals is therefore important for the assessment of possible zoonotic risks. There may also be issues in relation to the degree of co-selection arising from the use of tetracyclines in animals.

Daptomycin: Daptomycin is classified as a lipopeptide. It is a natural compound active only against Gram-positive bacteria. This antimicrobial is not used in animals but considered of critical importance in human medicine. Knowledge of the occurrence of resistance in bacteria from animals is therefore important for assessment of zoonotic risks.

5.2. Epidemiological cut-off values

As recently pointed out (EFSA, 2012a and 2012c), EUCAST ECOFFs values are to be used, when available, as the interpretative criteria to define microbiological resistance, thus separating the wild-type population from a population with acquired or mutational resistance towards a given antimicrobial substance. When no EUCAST data are available for a given antimicrobial substance, then criteria reviewed by the EURL-AMR may be used. It is proposed that EUCAST ECOFFs are maintained in the EU legislation for the purpose of harmonisation, optimally with synchronisation of their periodic updates in the legislation.

5.3. Recommended concentration ranges to be tested

Recommendations on the optimum concentration range to be tested for each antimicrobial should take into account the feasibility and cost-effectiveness of such testing. More specifically, the space available on a 96-well plate has been considered when outlining proposals intended to favour the testing of more substances rather than extended ranges of concentrations. The proposed concentration ranges to be tested should cover both EUCAST ECOFFs and clinical breakpoints so as to ensure comparability with human isolates. It was also considered desirable to test concentrations corresponding to the 'left' side of wild-type distributions (those lower than the modal concentrations), but this does not provide any additional information, since these values are meant to be constant over time. Rather it was considered more relevant to completely encompass the distributions of MICs for isolates with acquired reduced susceptibility.

5.4. Synoptic tables on antimicrobials, ECOFFs and concentration ranges recommended

In light of the above, proposals for a harmonised set of antimicrobial substances to be used for testing of susceptibility in *S. aureus*/MRSA are presented in Tables 4 and 5. The proposals are mainly based on the distributions of MICs available on the EUCAST website. The tables show three different proposed ranges for each antimicrobial. The "*Optimum*" range is set to encompass the complete MIC distribution, including that for the wild-type and the subpopulation with acquired decreased susceptibility/resistance. The "*Advised*" range is set to give as good coverage as possible of the complete MIC distribution. This range, however, takes into account the room available on a 96-well plate by omitting the lower MICs for the wild-type but covering the MICs of the subpopulation with acquired reduced susceptibility/resistance. In the "*Minimum*" range the intention is to encompass the distribution from modal MIC for wild-type isolates and most of the subpopulation with acquired reduced susceptibility/resistance (non-wild-type population).

Table 3: Proposed panel of antimicrobial substances, EUCAST epidemiological cut-off values (ECOFFs) and clinical breakpoints and concentration ranges to be tested in all MRSA isolates

Antimicrobial	Species	EUCAST values[a] (in mg/L)		Range of concentrations (in mg/L)			
				Current recommendation	New recommendation (no of wells in brackets)		
		ECOFF	Clinical R breakpoint		Optimal	Advised	Minimum
Cefoxitin	*S. aureus*	>4	>4	none	4-64 (5)	4-32 (4)	4-32 (4)
Chloramphenicol	*S. aureus*	>16	>8	none	1-128 (8)	4-64 (5)	4-32 (4)
Ciprofloxacin	*S. aureus*	>1	>1	none	0.06-256 (13)	0.25-16 (7)	0.5-8 (5)
Clindamycin	*S. aureus*	>0.25	>0.5	none	0.03-256 (14)	0.12-8 (7)	0.12-8 (7)
Erythromycin	*S. aureus*	>1	>2	none	0.06-512 (14)	0.5-64 (8)	0.5-32 (7)
Gentamicin	*S. aureus*	>2	-	none	0.06-64 (11)	0.5-32 (7)	0.5-16 (6)
Linezolid	*S. aureus*	>4	>4	none	0.25-8 (6)	1-8 (4)	2-8 (3)
Mupirocin	*S. aureus*	>1	>256	none	0.06-512 (14)	0.25-256 (11)[b]	0.25-8 (6)
Quinupristin/dalfopristin	*S. aureus*	>1	>2	none	0.06-4 (7)	0.25-4 (5)	0.24-4 (5)
Sulfamethoxazole/trimethoprim[c]	*S. aureus*	>0.5	>4	none	0.03-4 (8)	0.12-4 (6)	0.25-4 (5)
Tetracycline	*S. aureus*	>1	>2	none	0.12-256 (12)	0.5-64 (8)	0.5-8 (5)
Tiamulin	*S. aureus*	>2	ND	none	0.25-64 (9)	0.5-8 (5)	1-8 (4)
Vancomycin	*S. aureus*	>2	>2	none	0.25-8 (6)	1-8 (4)	1-8 (4)

ND, not determined.

(a): June 2012.

(b): The recommended concentration range of 0.25-256 mg/L enables high-and low-level resistance to be distinguished.

(c): It may be considered to test sulfamethoxazole and trimethoprim separately. In that case, the following values may be used to test for susceptibility:
Sulfamethoxazole: ECOFF: >128, Clinical breakpoint: >1024 (CLSI), Optimal range: 4-2048 (10), Advised range: 32-1024 (5), Minimum range: 128-1024 (4).
Trimethoprim: ECOFF: >2, Clinical breakpoint: >4, Optimal range: 0.25-512 (12), Advised range: 0.5-32 (7), Minimum range: 1-16 (5).

Table 4: Proposed panel of optional antimicrobial substances, EUCAST epidemiological cut-off values (ECOFFs) and clinical breakpoints and concentration ranges to be tested in all MRSA isolates

Antimicrobial	**Species**	**EUCAST values**[(a)] (in mg/L)		**Range of concentrations** (in mg/L)			
				Current recommendation	**New recommendation** (no of wells in brackets)		
		ECOFF	**Clinical R breakpoint**		**Optimal**	**Advised**	**Minimum**
Ceftobiprole	*S. aureus*	NA	NA	none	0.25-8 (6)	0.5-8 (5)	2-8 (3)
Kanamycin	*S. aureus*	>8	ND	none	0.25-32 (8)	1-32 (6)	2-32 (5)
Tigecycline	*S. aureus*	>0.5	>0.5	none	0.03-1 (6)	0.12-1 (4)	0.25-1 (3)
Fusidic acid	*S. aureus*	>0.5	>1	none	0.06-16 (9)	0.12-8 (7)	0.12-4 (6)
Daptomycin	*S. aureus*	>1	>1	none	0.06-16 (9)	0.25-8 (6)	0.5-4 (4)

NA, not yet publicly available; ND, not determined.

(a): June 2012.

5.5. Further testing of MRSA isolates

5.5.1. Detection of constitutive and inducible resistance to macrolides, lincosamides and streptogramins in *S. aureus*

MRSA ST398 isolates from pigs in Portugal have been reported as showing an unusual resistance phenotype, with resistance to clindamycin and susceptibility to erythromycin (Kadlec et al., 2010). This resistance is related to the presence of *vga*(A) or *vga*(C) genes which confer resistance to lincosamides, pleuromutilins and streptogramin A antimicrobials. Further examination of isolates which conversely show erythromycin but not clindamycin resistance using the D-test is suggested, because the animal species in which MRSA has been detected (for example, pigs) may be treated with both macrolide or lincosamide compounds and this may provide additional useful information when investigating possible epidemiological associations between isolates. The proposed monitoring is not performed for therapeutic purposes, but it is also useful to know whether animal-associated strains which are erythromycin resistant possess inducible clindamycin resistance.

Resistance to macrolides, lincosamides and streptogramins in *S. aureus* is mainly conferred by the macrolide, lincosamide and streptogramin B system (MLSB system) through the possession of *erm* resistance genes. These *erm* genes may be expressed inducibly or constitutively (LeClercq and Courvalin, 1991a and 1991b). Erythromycin, a 14-membered macrolide, induces the *erm* genes in *S. aureus*, whereas clindamycin and 16-membered macrolides are not inducers. Thus, MLSB-inducible *S. aureus* shows resistance to erythromycin but not to clindamycin, whereas MLSB-constitutive *S. aureus* is resistant to both erythromycin and clindamycin (Livermore et al., 2001).

In MLSB-inducible *S. aureus* isolates, erythromycin antagonises clindamycin, and this can be observed in double disc diffusion tests using erythromycin and clindamycin discs; the zone of inhibition around the clindamycin disc is reduced ("flattened") adjacent to the erythromycin disc in this procedure, commonly referred to as the "D-test" (Livermore et al., 2001; Levin et al., 2005).

It is proposed that the D-test should be used to provide additional useful epidemiological information on MRSA isolates which are resistant to erythromycin but susceptible to clindamycin, and should, therefore, be performed on all or a convenient proportion of MRSA isolates, depending on the number of isolates involved and resources available. The results should be reported to the EFSA as D-test positive (inducible resistance detected) or negative (inducible resistance not detected). The occurrence of an inducible or constitutive MLSB phenotype in *S. aureus* isolates will be inferred from the measured MICs of erythromycin and clindamycin and the result of the D-test. Resistance to both erythromycin and clindamycin will be considered to imply a constitutive MLSB phenotype and resistance to erythromycin but not clindamycin with a positive D-test will be considered to indicate the inducible MLSB phenotype.

Performing the D-test provides optional additional information which may be used to characterise *S. aureus* isolates; it may also have clinical veterinary relevance in the case of bovine mastitis isolates. Where MSs have examined erythromycin-resistant clindamycin-susceptible isolates by D-test for inducible or constitutive resistance, then the results will be analysed as part of the collation of results.

5.5.2. Vancomycin susceptibility testing of *S. aureus*

Vancomycin-resistant MRSA was first reported in 2002 (United States Centers for Disease Control and Prevention (CDC), 2002); however, reduced susceptibility to vancomycin in MRSA had previously been reported in Japan in 1997 (Hiramatsu et al., 1997).

The current EUCAST clinical breakpoints for vancomycin in *S. aureus* are sensitive <2 mg/L and resistant >2 mg/L. However, intermediate breakpoints have also been available from the USA National Committee for Clinical Laboratory Standards (NCCLS)[15], and these covered MICs of 8-

[15] Currently known as the Clinical Laboratory Standards Institute (CLSI).

16 mg/L (Brown et al., 2005). The existence of an intermediate category of resistance led to the development of a nomenclature in which *S. aureus* could be classified as vancomycin-susceptible *S. aureus* (VSSA), vancomycin-intermediate *S. aureus* (VISA) or vancomycin-resistant *S. aureus* (VRSA). However, detection of low-level or intermediate resistance or reduced susceptibility is problematic and, in the case of these isolates, MIC testing does not clearly differentiate between the categories of VSSA and VISA and the additional category heterogeneous vancomycin-intermediate *S. aureus* (hVISA). Heterogeneous low-level resistance to vancomycin is displayed by hVISA isolates, for which the MIC of vancomycin may be <2 mg/L ("sensitive"), but in the case of these isolates, a subpopulation of cells present within the expanding clone is able to grow in the presence of higher vancomycin concentrations. Human infections caused by hVISA appear to respond less well to vancomycin treatment (Wootton et al., 2005).

Screening methods have been developed to detect VISA and hVISA isolates and thereby identify reduced susceptibility to vancomycin; the method using Mueller-Hinton agar with 5 mg/L teicoplanin has been reported to perform well (Wootton et al., 2007). The population analysis profile (PAP) method is used to confirm the presence of heterogeneous resistance (Wootton et al., 2001).

High-level vancomycin resistance is usually related to acquisition of vancomycin resistance genes. Acquisition of *vanA* genes by vancomycin-resistant *S. aureus* is extremely rare and usually confers high-level resistance to vancomycin, with MIC values >32 mg/L.

Because MIC testing does not clearly differentiate between the categories of VSSA, VISA and the further category hVISA, and because of the inherent one dilution well variability observed with MIC testing, it is proposed that only *S. aureus* isolates for which the MIC of vancomycin is >8 mg/L should be retained for further testing, which should comprise molecular examination for vancomycin resistance genes. The highest vancomycin concentration recommended in the dilution series is 8 mg/L (see section 5.4). The PAP method is specialised and should be applied only where there are *spa* or multilocus sequence types of MRSA from animals implicated in human disease and where consultation at the national level with medical colleagues indicates that such detailed examination is warranted.

6. Recommendations on the format for the collection and reporting of data on MRSA

6.1. Current reporting of data on MRSA

Although reporting of MRSA antimicrobial resistance data is not currently compulsory, several MSs report data on the prevalence of MRSA in animals and food, as well as aggregated antimicrobial resistance data, within the framework of the annual zoonoses reporting (EFSA and ECDC, 2012). It should be noted that the methods for collecting and testing samples are not harmonised, and as a result MSs may use differing procedures with a concomitant reduction in the comparability of results between MSs (EFSA and ECDC, 2012). In 2010, two MSs reported data on the occurrence of MRSA in food, while seven MSs and one non-MS reported MRSA data for animals. Moreover, data on multiple antibiotic resistance of MRSA isolates have been reported by two countries (EFSA and ECDC, 2012). Molecular typing results were also reported on several occasions.

6.2. General provisions for harmonised reporting of data on MRSA

The competent authority responsible for the preparation of the annual national report on zoonoses pursuant to Article 9 of Directive 2003/99/EC should ensure that the results of the monitoring of the prevalence, genetic diversity and antimicrobial resistance of MRSA are collected, evaluated and reported in the annual zoonoses report to the Commission and the EFSA.

6.3. Collection and reporting of MRSA isolate-based data

The information to be reported on the monitoring of the prevalence, genetic diversity and antimicrobial resistance of MRSA should be reported at sample/isolate level. The collection and reporting of data on MRSA at the isolate level enables more in-depth scientific analysis. In particular, it would be beneficial for investigating multi-resistance patterns and reporting subtypes and performing analysis of genetic diversity, as well as evaluating geographical progression over time, conducting retrospective analysis and assisting in source attribution. To this end, it is proposed to complement the AMR isolate-based data model specifically designed by the EFSA recently (EFSA, 2012b) and currently used, after slight modifications, for the collection and transmission of isolate-based data from the reporting year 2011 (EFSA, 2012b), to collect results from MRSA typing and subtyping. Isolate-based data will be used to generate Extensible Markup Language (XML)/Excel files that can be submitted by the MSs as part of their national reports.

The components of the model and the information to be reported by MSs are outlined in Table 6. They comprise the following broad categories: description of the monitoring programme and the sampling performed, information about the laboratory, and monitoring typing and antimicrobial susceptibility results. Information and data reported related to the matrix (e.g. animal populations, food categories), sampling unit, sample type, as well as the total number of biological samples and epidemiological units tested should inform on the MRSA prevalence. In particular, the data model includes the following specific variables to report detailed information on the MRSA typing:

- *spa*-type: the *spa*-type of the isolate (alphanumeric code)
- Clonal complex: the clonal complex of the isolate (alphanumeric code)
- Clonal complex origin: dichotomous variable: 'Performed' / 'Inferred', indicating whether the clonal complex has been inferred from the *spa* type or derived from a specific test, such as MLST typing.
- Multilocus Sequence type: the MLST of the isolate (alphanumeric code)
- MLST origin: dichotomous variable: 'Performed' / 'Inferred', indicating whether the MLST has been inferred from the *spa* type or derived from MLST typing.
- Repeat sequence: specific repeated DNA sequence of the variable region of the *spa* gene (alphanumeric code), to be reported in case of novel *spa*-type.
- D-test results: dichotomous variable: 'positive' vs. 'negative'
- Presence of PVL-related genes: dichotomous variable: 'positive' vs. 'negative'
- Presence of vancomycin resistance genes: dichotomous variable: 'positive' vs. 'negative'

Table 5: Information included in the data model for the reporting of isolate-based MRSA data

General information and identification of the isolate

- Result Code
- Reporting Year
- Reporting Country
- Language

Information about type and source of samples and isolates

- Zoonotic agent (in this case MRSA)
- Matrix (e.g. investigated animal populations, food categories, etc.)

Information about the sampling performed

- Sampling unit
- Sampling stage
- Sample type
- Sampling context
- Sampler
- Programme Code
- Sampling strategy
- Sampling details
- Area of Sampling
- Total number of biological samples tested
- Total number of epidemiological units tested

Information about the laboratories

- Laboratory(ies) Identification Code
- Laboratory(ies) Isolate Code

Information about the sampling and isolation

- Sampling: Year/month/day
- Isolation: Year/month/day

Information about the typing of MRSA

- *spa*-type (mandatory)
- Clonal complex
- Clonal complex origin: Performed vs. Inferred
- Multilocus sequence type (MLST type)
- MLST origin: Performed vs. Inferred
- Repeat sequence (for new sequences)
- D-test results
- Presence of PVL-related genes
- Presence of vancomycin resistance genes

Information about the method for antimicrobial susceptibility testing

- Susceptibility: Test Year/month/day
- Method
- Antimicrobial substance
- Epidemiological cut-off value

Information about dilution method

- Lowest
- Highest
- MIC value

Additional information

- Comment

CONCLUSIONS AND RECOMMENDATIONS

ToR 1. Provide detailed guidance on the monitoring of MRSA: food animal species and/or foodstuffs and methodologies which should be considered as most relevant for antimicrobial resistance (AMR) monitoring from a public health perspective, taking into account AMR mechanisms;

- A number of food-producing animals have been acknowledged as a possible source of MRSA colonisation in farmers, veterinarians, and their families, through direct or indirect contact with animals and their environment. The recognised livestock-associated MRSA (LA-MRSA) strain appears to be primarily acquired by humans through occupational exposure. In addition, MRSA in production animals may present a hazard for human healthcare systems because of the potential introduction of the bacteria into healthcare facilities via colonised livestock professionals.

- The role of food as a source of human colonisation or infection with MRSA is presently considered to be low. Epidemiological studies have shown that LA-MRSA is fairly infrequent in people without direct or indirect contact with livestock. However, MRSA has been shown to evolve continuously, and changes in virulence and transmissibility may also occur in the future. For this reason, monitoring the diversity of MRSA in livestock is important.

- The prevalence and characteristics of LA-MRSA in animals and food need to be monitored regularly to assess and detect changes in prevalence and in MRSA subtypes. Considering the predominant route of transmission, monitoring in primary production, including at the slaughterhouse, seems pivotal. Monitoring MRSA in meat, raw milk and raw milk products may also help with the assessment of exposure of the consumers via this route, although it is at present considered of minor importance.

- MRSA monitoring in food-producing animals and food derived thereof should optimally be complemented by monitoring in companion animals, such as dogs and cats, which have proved to play a role in the diffusion and exchange of MRSA with humans. However, this monitoring is beyond the remit of the framework legislation and the mandate received and has therefore not been considered, although EFSA could collect data if provided on a voluntary basis by MSs.

- Thus, monitoring the prevalence and diversity, including resistance profile, of MRSA in livestock populations is considered to be the primary objective of the present proposed monitoring programme.

- Knowledge of the different MRSA subtypes prevalent in food-producing animals and their characteristics (virulence and resistance profiles) enables comparison with the MRSA strains isolated in humans, which may shed light on the transmission and diffusion of strains among the human and animal populations. As such, the monitoring programme would allow the detection of the emergence of LA-MRSA in the human populations and also, conversely, the potential emergence of community-acquired MRSA in livestock populations.

- Accounting for the most recent scientific results on MRSA, the following definition of MRSA is proposed for the purpose of the harmonised monitoring of MRSA in animals and food in the EU: *S. aureus* harbouring either the *mecA* or *mecC* gene or, if negative for these genes, phenotypically resistant to cefoxitin. The goal of monitoring is to characterise MRSA according to sub-types, such as *spa*-types, CCs etc.), and the presence of virulence genes of importance.

- Priorities for the monitoring of MRSA in food-producing animals have been set up from a public health perspective, focusing on the animal populations considered to be MRSA reservoirs. As a consequence of this approach, a list of food-producing animal populations has been drawn up for which consistent monitoring is recommended every third year on a rotating basis. This list comprises:
    - broilers,
    - fattening pigs,
    - dairy cattle.

- The concept of a threshold (based on tonnage of animals slaughtered) has been introduced to establish proposals for mandatory monitoring in some animal populations. On the basis of this approach, consistent monitoring for MRSA is recommended in fattening turkeys and calves under 1 year of age in countries where production exceeds 10 million tonnes slaughtered/year. Monitoring should be carried out within the framework of existing national control programmes, whenever this is possible. In particular, it is proposed to link MRSA monitoring with the mandatory surveillance of *Salmonella* in broiler and turkey flocks in the EU.

- A second list of food-producing animal populations, consisting of animals that may play a role in potential (sometimes clonal) diffusion of MRSA, in which monitoring of MRSA can be performed on a voluntary basis, has also been drawn up. This list comprises:
    - breeding flocks of *Gallus gallus* from the meat sector,
    - breeding flocks of turkeys,
    - beef animals,
    - breeders of pigs,
    - horses.

- To fulfil the primary objective of assessing the diversity of LA-MRSA strains present in animal populations, monitoring of the food chain at the level of the slaughterhouse is considered to be sufficient and is recommended for fattening pigs and calves under 1 year. Monitoring at the slaughterhouse will increase the detection rate of MRSA as it is known that cross-contamination occurs (between animals from the same source but also animals from different sources) during transport and lairage, thus inducing an amplifier effect. In the case of poultry and dairy cattle, on-farm sampling is recommended to facilitate sample collection.

- Sampling the pig population at the slaughterhouse level is considered the most cost-effective and adequate approach in those MSs where the prevalence is low to very low. For those MSs that have already detected a high prevalence of MRSA in their pig populations and wish to estimate this prevalence at the farm level and/or better assess the epidemiology of MRSA, monitoring pigs at the farm level may be of specific interest.

- It is acknowledged that the role of food as a source of human colonisation or infection with MRSA is presently considered to be low. For this reason it is proposed that monitoring of MRSA be performed only on a voluntary basis in the following food categories:
    - broiler meat,
    - turkey meat,
    - pork,
    - beef,
    - veal,
    - raw milk and products derived from raw milk.

- Since the MRSA prevalence in the monitored animal populations is not expected to change rapidly over a 1- to 2-year period, monitoring every 3 years and covering the different animal populations targeted on a rotating basis should be sufficient to determine the prevailing situation and the diversity of strains prevalent in each particular country and at EU level. It would however be desirable that all MSs conduct the monitoring in the same animal populations in the same year.

- In providing recommendations regarding the sampling stage, careful consideration has been given to the possibility of using samples derived from other existing monitoring schemes, whenever possible. Thus, it is recommended that environmental samples similar to those collected in the framework of the mandatory *Salmonella* control programmes in poultry (broilers and fattening turkeys) are used for the monitoring of MRSA. Care should only be taken to collect one additional pair of boot swabs for the purpose of MRSA monitoring, since laboratory procedures are very different for *Salmonella* and MRSA. Nasal swab sampling at the slaughterhouse is proposed for fattening veal calves (under 1 year of age) and slaughter pigs. In certain MSs, the calf population to be monitored for MRSA may also comprise fattening calves older than 1 year. With respect to monitoring of MRSA on pig farms, nasal swab sampling is also proposed with a special emphasis on fattening pigs during the last two months of the fattening period. For dairy cows, it is recommended that bulk tank milk sampling be carried out on the dairy farm.

- Regarding the voluntary monitoring of MRSA in breeding animals of pigs, *Gallus gallus* and turkeys, and in beef animals and horses on-farm sampling is also recommended. The above recommendations regarding the nature of the samples to be collected apply likewise.

- With regard to the sampling of food, a greater level of flexibility is offered, leaving it up to each individual MS to decide whether this should be performed at retail or at processing/cutting plant level. Monitoring of domestic production may be complemented by monitoring of imported food.

- Formal randomised sampling strategies should preferentially be applied, allowing for proper statistical data analysis and reducing the effect of sampling bias. Random sampling in each targeted animal population ensures the representativeness of the entire population, and reflects the variability in managerial and hygienic practices in holdings and different country regions. It is important that the bacterial isolates originate from healthy animals sampled from randomly selected holdings/flocks or randomly selected within the slaughterhouses. An approximately uniform distribution of the collected samples over the year enables the different seasons to be covered.

- As there is significant diversity between MSs in MRSA prevalence in the targeted food-producing animal populations, it is acknowledged that the sample size should be adapted to the local epidemiological situation and calculated at the MS level. The target sample size may account for the purpose of assessing prevalence and/or determining a trend. With regard to the minimum MRSA isolate sample size for monitoring antimicrobial susceptibility, the figure of 170 isolates per year is recommended as an optimal isolate sample size, although this number of isolates may be difficult to achieve in food production sectors with low to medium MRSA prevalence. In that case, a sample of minimum size should be defined and collected. Sample size may be calculated with the purpose of investigating that MRSA prevalence is likely not above an expected level in defined animal populations.

- As regards the analytical methods to be used, the first step is to isolate presumptive MRSA, MRSA is then confirmed by the presence of *mecA* or *mecC* by multiplex PCR or, if negative for these genes, by phenotypical test for resistance to cefoxitin. MRSA confirmed isolates are mandatorily further *spa* typed in order to determine the corresponding clonal complex (CC).

Isolates where no CC can be determined based on the *spa*-type should be MLST typed. Further analytical tests can be performed to further characterise isolates.

- Identification of the PVL toxin is recommended so that community-acquired MRSA can be detected in livestock populations, if present.

ToR 2. Consider antimicrobials, epidemiological cut-off values and recommended optimum concentration ranges to be used for susceptibility testing of MRSA isolates;

- The proposed lists of antimicrobials to be included in AMR monitoring in MRSA are as follows:
  - Recommended set: cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, linezolid, mupirocin, quinupristin/dalfopristin, sulfamethoxazole/trimethoprim, tetracycline, tiamulin and vancomycin.
  - Optional set: ceftobiprole, kanamycin, tigecycline, fusidic acid and daptomycin.

- In the interpretation of resistance, the use of the EUCAST epidemiological cut-off values is recommended, whenever available, and should be included in the EU legislation for harmonisation purposes. A periodic revision of the corresponding legislation should therefore be envisaged to ensure that updates to the values are adequately reflected in the legislation.

- When proposing amendments to the existing panel of antimicrobials, the room available in a 96-well plate was considered. Accordingly, the proposed number of substances to be tested had to be offset by a limited range of concentrations tested for some of these substances.

- To this end, proposals have been made defining optimal, advised and minimum concentration ranges to be tested. Both the EUCAST epidemiological cut-off values and the clinical breakpoints are, however, included in the minimum range, so that the data can still be analysed and also compared with MRSA strains isolated from humans.

- Further optional testing of MRSA isolates is also proposed for the detection of constitutive and inducible resistance to macrolides, lincosamides and streptogramins.

- Since vancomycin is an important antibiotic for the treatment of MRSA infections in humans, any detected resistance to vancomycin should be subject to further characterisation (PVL-toxin determination).

ToR 3. Indicate the best format for the collection and reporting of data

- Analyses on multi-resistance, specific co-resistance patterns and association between resistance traits cannot be performed on the currently available dataset deriving from reporting of aggregated AMR data. In order to perform such analyses, information needs to be collected with a greater level of granularity, and data must be reported at the level of each bacterial isolate tested for antimicrobial susceptibility and correlated with *spa*-types.

- It is expected that transmission of data at the level of the isolates would facilitate the reporting of detailed epidemiological information and would consequently allow performance of more detailed analyses for inclusion in the EU Summary Report on AMR.

- Given the public health relevance of the emergence of multi-resistant bacteria, it is therefore strongly recommended that MRSA data collection is performed at isolate level by the MSs and other reporting countries.

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APPENDIX

Overview of complementary typing methods of MRSA of epidemiological interest

- **SCC*mec* typing**

The SCC*mec* typing is based on a basic classification which has been designed based on the identification of the main SCC*mec* elements which are present: the *ccr* (chromosomal cassette recombinase) gene complexes and the *mecA* class define the basic types (Table 7).

Table 7: SCC*mec* types currently identified according to International Working Group on the Classification of SCC*mec* elements (IWG-SCC).

SCC*mec* types	*ccr* gene complexes	*mec* gene complexes	Strains
I	1 (A1B1)	B	NCTC10442, COL
II	2 (A2B2)	A	N315, Mu50, Mu3, MRSA252, JH1, JH9
III	3 (A3B3)	A	85/2082
IV	2 (A2B2)	B	CA05, MW2, 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, E-MRSA-15, JCSC6668, JCSC6670
V	5 (C1)	C2	WIS(WBG8318), TSGH17, PM1,
VI	4 (A4B4)	B	HDE288
VII	5 (C1)	C1	JCSC6082
VIII	4 (A4B4)	A	C10682, BK20781
IX	1 (A1B1)	C2	JCSC6943
X	7 (A1B6)	C1	JCSC6945
XI	8 (A1B3)	E	LGA251

Several authors have designed methods for allowing the classification of SCC*mec* types using multiplex PCR methods and they vary in the targets amplified and types that can be classified. The most commonly used methods are those developed by Kondo et al. (2007), which are based on two main multiplex PCR reactions for the typing of *ccr* complex and *mec* class and additional multiplex PCR schemes for subtyping. However, this typing scheme is not applicable for the SCC*mec* types (VII, X, XI) containing *ccr* elements 6-8 and *mecA* classes C1, D and E. Additional methods are available, designed on one hand by Milheiriço et al. (2007), and based on another PCR multiplex design, for the identification of SCC*mec* elements I-VI and developed on the other hand by Zhang et al. (2005) and McClure et al. (2010) for typing SCC*mec* elements type I-V and type VIII.

SCC*mec* subtyping: Many different structures, including insertion sequences and transposons, have been identified among the major SCC*mec* types in regions other than the *mec* gene complex and *ccr* gene complex, e.g. in the J regions. Each SCC*mec* type has therefore been further classified into subtypes based on the polymorphisms or variations in J regions within the same *ccr* gene complex and *mec* gene complex combination. Subtyping the SCC*mec* elements is sometimes necessary to define the elements present, for example the cassettes within SCC*mec* complex IV, which are among those frequently found among livestock-associated strains. Further testing schemes for subtyping of *ccr* elements and for classification of variants and composite cassettes and for subtyping of *ccr* elements are also available (Higuchi et al., 2008 – *ccrB* typing: http://www.ccrbtyping.net/).

The novel *mecC* gene has so far been found only in the SCC*mec* elements of type XI and can be typed using a different method described by García-Álvarez et al. (2011).

In addition to the PCR methods, genotyping arrays such as the Identibac array (http://identibac.com/en/assay-principle-products/products-available/s-aureus-genotyping.html) for *S. aureus* (commercialized by Alere Technologies, Germany) also enable the detection of structures within the SCC*mec* element as well as of resistance and virulence genes. Thus, such arrays might provide a typing result, but they can only be performed if specific array readers are available and are limited to the probes available in the array.

- **Analysis of chromosomal DNA by Pulsed-Field Gel Electrophoresis (PFGE)**

When using PFGE (see Glossary) on MRSA isolates, it is advantageous to know the CC type as CC398 DNA cannot be cut by the restriction endonuclease *Sma*I. Therefore, different protocols are used for CC398 and non-CC398 with regard to the enzyme used for digestion of the DNA. PFGE has been used for typing of MRSA non-CC398 and CC398 in several studies (Argudín et al., 2010; Bosch et al., 2010; Gómez-Sanz et al., 2010). It is recommended that some laboratories are appointed to run the analysis as equipment varies between laboratories, and this can make results difficult to compare. For example, differences in electrophoresis conditions can complicate the comparison between isolates submitted to electrophoresis in different gels.

- **Whole-genome sequencing (WGS)**

At present, there are some limitations to the use of WGS for *spa*-typing and typing of SCC*mec*, as repeat regions in these areas may cause problems with the sequencing and interpreting. However, web-based tools are available for MLST, species identification and the search for resistance genes (ResFinder database) at the Center of Genomic epidemiology (http://www.genomicepidemiology.org). Currently, not all laboratories can perform WGS, but several companies provide this service and the interpretation of the results can be done in collaboration with other laboratories or the EURL-AMR.

GLOSSARY

Clonal complex (CC)	The *S. aureus* population including MRSA consists of different clonal lineages, also called clonal complexes. To determine which lineage an isolate belongs to, the sequence type has to be determined by a method called Multi Locus Sequence Typing. Closely related sequence types (STs) are grouped into the same CC by the web-based computer software called eBURST.
Multi Locus Sequence Typing (MLST)	Multi Locus Sequence Typing is a molecular typing method that allows determination of nucleotide differences between isolates of microbes. In the case of *S. aureus*, including MRSA strains, it is used to identify the seven housekeeping genes present in all *S. aureus* strains: *arcC* (coding for carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase) and *yqi* (acetyl coenzyme A acetyltransferase). The method was first described by Enright et al. (2000). This technique involves the sequencing of defined sections of those seven genes, and their comparison using a publicly available database (www.mlst.net). MLST enables the assignment of sequence types to each MRSA.
Pulsed-Field Gel Electrophoresis (PFGE)	This technique is based on the analysis of bacterial chromosomal DNA by digestion with restriction endonucleases that recognise few sites along the chromosome, generating large fragments of DNA (10-800 kb). Consequently, PFGE allows for the comparison of chromosomal DNA with much simpler profiles than those generated by high-frequency restriction endonucleases. All bacteria, including MRSA, can theoretically be typed by PFGE, and the results are highly reproducible. The restriction endonuclease showing the best performance in MRSA among those tested is *Sma*I. Standardised interpretation schemes have been proposed in order to determine the genetic relationship between strains.
Phylogenetic analysis	Analysis of the evolution and of relations among various groups of organisms (e.g. species, populations), which is determined by molecular sequencing data and morphological data matrices.
RIDOM StaphType Database (www.SpaServer.ridom.de)	Single-locus DNA sequencing of the repeat region of the *Staphylococcus* protein A gene (*spa*) can be used for reliable, accurate and discriminatory typing of MRSA. Repeats are assigned a numerical code and the *spa*-type is deduced from the order of specific repeats. However, *spa*-typing was hampered in the past by the lack of a consensus on assignments of new *spa*-repeats and *spa*-types. This SpaServer can be used to collate and harmonise data from various geographic regions. This website (SpaServer.ridom.de) is freely accessible to internet users and the *spa*-repeat sequences and the *spa*-types can be downloaded. Chromatograms of new *spa*-repeats and/or -types can be submitted online for inclusion in the reference database.

SCC*mec* typing	The emergence of methicillin-resistant staphylococcal lineages is due to the acquisition and insertion of the so-called Staphylococcal Chromosome Cassette mec (SCC*mec*) elements into the chromosome of susceptible strains. SCC*mec* elements are highly diverse in their structural organization and genetic content and therefore can be classified into types and subtypes. The typing of the SCC*mec* cassette based on the determination of its types and subtypes is normally performed by PCR amplification of the elements present within the SCC*mec* element.
Spa-typing	A molecular typing method used for the subtyping of *S. aureus* including MRSA and subsequently for identifying MRSA lineage. This technique involves PCR amplification and sequencing of the variable region of the protein A (*spa*) gene, which encodes the staphylococcal protein A located on the chromosome of all *S. aureus* isolates. The variable region of the *spa* gene consists of specific repeated DNA sequences (called "repeats"). The *spa*-typing method assigns alpha-numeric codes to different repeats and, based on their order and composition, *spa* repeat sequences are automatically assigned a *spa*-type by submission to the RIDOM StaphType Database (www.SpaServer.ridom.de). The most likely sequence type of new s*pa*-types can often be inferred by comparing them with well-defined *spa*-types with close *spa* repeat homology for which the ST has already been determined by MLST. MRSA with the same *spa*-type will, in most cases, belong to the same sequence type/clonal complex.

ABBREVIATIONS

AMR:	Antimicrobial Resistance
ATCC:	American Type Culture Collection
BURP:	Based Upon Repeat Pattern
CC:	clonal complex
ccr:	chromosomal cassette recombinase
CDC:	United States Centers for Disease Control and Prevention
CLSI:	Clinical Laboratory Standards Institute
CNS:	Coagulase-Negative Staphylococci
DNA:	Deoxyribonucleic Acid
EC:	European Commission
ECOFFs:	Epidemiological Cut-Off
EFSA:	European Food Safety Authority
EQAS:	External Quality Assessment Scheme
EU:	European Union
EUCAST:	European Committee on Antimicrobial Susceptibility Testing
EURL-AMR:	European Union Reference Laboratory for AMR
EUSR:	European Union Summary Report
FDA:	Food and Drug Administration
GRD:	Glycopeptide Resistance Detection
IWG-SCC:	International Working Group on the Classification of SCCmec elements
LA-MRSA:	Livestock-Associated Meticillin (or methicillin)-Resistant Staphylococcus aureus
MIC:	Minimum Inhibitory Concentration
MLSB resistance:	Cross-resistance to all macrolides as well as to lincosamides and streptogramins of the B type
MLST:	Multi Locus Sequence Typing
MRSA:	Meticillin (or methicillin)-Resistant Staphylococcus aureus
MSSA:	Meticillin (or methicillin)-Sensitive Staphylococcus aureus

MS(s):	Member State(s)
NaCl:	sodium chloride
NCCLS:	United States National Committee for Clinical Laboratory Standards
NRL:	National Reference Laboratory
PAP:	Population Analysis Profile
PCR:	Polymerase Chain Reaction
PFGE:	Pulsed-Field Gel Electrophoresis
PVL:	Panton-Valentine Leukocidin
QA:	Quality Assurance
SCCmec:	Stapylococcal cassette chromosome mecS/I/R: categorisation of S. aureus strains into susceptible, intermediately-resistant or resistant to vancomycin
ST398:	multilocus sequence type 398
VISA:	Vancomycin-Intermediate S. aureus
hVISA:	heterogeneous Vancomycin-Intermediate
VRSA:	Vancomycin-Resistant S. aureus
VSSA:	Vancomycin-Susceptible S. aureus
WGS:	Whole-Genome Sequencing
WHO:	World Health Organisation
XML:	eXtensible Markup Language