Executive Summary:

Methicillin-resistant Staphylococcus aureus (MRSA) is a major pathogen and important public health problem. CONCORD (CONtrol of COmmunity-acquired MRSA: Rationale and Development of counteractions) is a consortium of eight research institutes and one company aimed at finding explanations why MRSA became successful in the community and among livestock (community and livestock-associated (CA- & LA-) MRSA), and explore options for interventions to reduce the load of MRSA or even eradicate MRSA from some reservoirs.

CONCORD collected a large set of European MRSA isolates. Typing showed a very high level of diversity among CA-MRSA in Europe. Our results suggest that some CA-MRSA types probably originated in Europe and others were imported. Besides the variation of types, closely related isolates can show differences in gene content and also expression of identical genes may vary. Nevertheless when the gene content of different MRSA are compared it is possible to group them in hospital-associated (HA-), LA-, and CA-MRSA.

Resistance to zinc is associated with methicillin resistance in LA-MRSA. This metal resistance gene was detected in three-quarters of European LA-MRSA suggesting that the use of zinc in animal production might have led to co-selection of methicillin resistance and may have contributed to the emergence of LA-MRSA. Other staphylococci than S. aureus which are present on farms may serve as a reservoir for the gene encoding methicillin resistance and thereby can contribute to the development of LA-MRSA. Genetic analysis showed that LA-MRSA carry a different set of genes encoding proteins that interfere with the host immune system than human adapted S. aureus types. CA-MRSA more often carry Panton-Valentine leukocidin (PVL) compared to HA-MRSA and methicillin-susceptible S. aureus (MSSA). PVL may contribute to the success of CA-MRSA. These virulence factors may become part of a vaccine. HA-MRSA have lost some capacity to produce proteins and thus fitness in favor for higher levels of resistance, enhancing success in hospitals. The reduced fitness makes them unable to compete in the community setting. CA-MRSA are identical to methicillin-susceptible S. aureus in this respect explaining their success in the community.

Two novel mathematical models to study MRSA transmission in different settings were developed: a LA-MRSA between-farm transmission model and a between-hospital transmission model of health care associated infections. Movement-induced transmission alone can yield a high probability of LA-MRSA persistence at very low prevalences. Due to the high volume and frequency of between-farm trade, LA-MRSA eradication may be difficult. There are currently no practical, low-cost, farm-based control strategies that will effectively tackle endemicity in the pig industry. Endemic prevalence levels obtained with 2008 movements are comparable with prevalence estimates from the European Food Safety Authority baseline survey. However, the model is not able to reproduce the results of more recent surveys in years 2009 and 2010. The referrals of patients play an important role in the spread of nosocomial infections. Each index hospital in England (the Netherlands) can affect, on average, up to a maximum of 106 (56) other hospitals, which correspond to 73% (57%) of the health care network. Employing an empirical approach a hospital priority list was built to aid in the selection of surveillance sentinels. The

model supports the traditional approach in the design of hospital-based surveillance systems. In the endemic regime that arises when implementing complete pathogen elimination from individual hospitals, high complexity hospitals re-acquire the nosocomial pathogen 4-6 times faster than low complexity ones. In Dutch hospitals, MRSA ST398 is less transmissible than non-ST3980. In Danish hospitals, CA-MRSA is less transmissible than HA-MRSA.

With the development of an in vitro pig nasal colonization model the interaction between S. aureus and a natural host can be studied. A pig phage therapy pilot experiment has been carried out and is under evaluation.

In conclusion CONCORD found explanations to explain the success of CA-MRSA and contributed to solutions of this problem.

Project Context and Objectives:

Introduction

Until the mid-1990s, methicillin-resistant Staphylococcus aureus (MRSA) were confined to the hospitals and effective control measures were known. However, MRSA has emerged as a community-associated pathogen. This includes the farm environment. The change in epidemiological and microbiological characteristics of MRSA is threatening the present control measures in hospitals. Community- and livestock-associated MRSA (CA-and LA-MRSA) has developed as an important cause of serious infections in the community and on farms. At present no strategies exist to combat these MRSA. Understanding the ecological success of CA-and LA-MRSA is essential to develop measures to control its spread in the community and on farms. CONCORD is aimed at explaining the ecological success in the community and the farm environment of CA- and LA-MRSA in contrast to hospital-associated MRSA (HA-MRSA) in order to facilitate the rationale and development of effective strategies against CA- and LA-MRSA.

Definition of MRSA: S. aureus isolates carrying the mecA gene. This gene is carried on a mobile genetic element (staphylococcal chromosome cassette or SCCmec) and encodes for a protein that confers methicillin resistance. Different SCCmec types, indicated by a Roman numeral, and subtypes, indicated by an additional letter, are known.

Definition of CA-MRSA: A uniform clinical definition of CA-MRSA does not exist. In this proposal we will use a definition based on genetic and epidemiological factors: CA-MRSA contains SCCmec type IV or V and are isolated from persons without a known health care association. Isolates with the genetic characteristics of CA-MRSA are now increasingly spreading and well established in hospitals and consequently might be called HA-MRSA. As a result the distinction between CA- and HA-MRSA is becoming increasingly blurred.

Definition of LA-MRSA: A definition of LA-MRSA does not exist and we will use a definition based on genetic and epidemiological data. LA-MRSA contain a SCCmec type IV or V and are isolated from animals, veterinarians or persons living on farms.

MRSA epidemiology

Methicillin-resistant S. aureus emerged in the early 1960s following the introduction of semi-synthetic penicillins such as methicillin. For nearly 30 years, MRSA were mainly restricted to hospitals where they had a selective advantage compared to drug-susceptible wild-type strains. Outside health care settings, MRSA were not able to spread, probably due to a fitness cost associated with the acquisition of the staphylococcal chromosome cassette carrying mecA

(SCCmec). In the 1990s MRSA were found in the community, in particular in (healthy) individuals who had no direct or indirect link with health care settings. The first epidemics of MRSA in patients without prior history of hospitalisations were described in the 1990s first in Australia and some years later in the United States. Since the beginning of this century CA-MRSA is increasing at an alarming rate and even has reached countries, which have not had major problems with health care associated MRSA-infections, such as Denmark and The Netherlands (Figure 1).

CA-MRSA mostly cause skin and soft tissue infections. Sometimes life-threatening infections and infections at uncommonly infected body sites are described. Necrotizing pneumonia is one of the most common severe infections. Patients with CA-MRSA seem to have more severe illness than those with other MRSA or methicillin-susceptible S. aureus (MSSA) strains. Analysis of mortality associated with MRSA suggests an increased mortality. In 2004 The Strategic Council on Resistance in Europe (SCORE) in which the UMCU participated calculated that blood stream infections caused by MRSA in Europe were annually responsible for an estimated 1300 additional deaths per year in the EU and 120 million in additional health care costs.

Clone diversity

Based on Multi-Locus Sequence Typing (MLST) studies most hospital-associated (HA-) MRSA belong to 5 clonal complexes and predominantly contain either SCCmec types I, II, or III, which encode methicillin resistance. CA-MRSA are genetically much more diverse and belong to a wide variety of clonal complexes and MLST sequence types (ST). CA-MRSA are also found in STs or clonal complexes (CCs) in which HA-MRSA are present. A striking difference with HA-MRSA is that CA-MRSA do not contain SCCmec I-III but SCCmec type IV or V.

Multi-Locus Sequence Typing (MLST): In MLST the DNA sequence of parts of 7 genes from different isolates are compared. When these are identical, isolates belong to the same sequence type (ST). Isolates differing in one gene belong to the same clonal complex (CC) and are related. The complexes are named after the ST after the supposed founder ST of the clonal complex. Usually this is the ST for which most isolates are known. E.g., ST398 which is the most ST among livestock-associated is believed the founder of CC398 in which several closely related other STs are present. Also the most isolates are known for ST398.

Spa types: All S. aureus isolates encode Protein A (gene name spa). This protein is anchored to the cell surface by a variable amino acid sequence. The variability of the genetic code for these anchor sequences between different strains is used for typing isolates. This typing method correlates with MLST.

Pulsed-Field Gel Electrophoresis (PFGE): A typing method in which the bacterial genetic material enzyme is cut by an enzyme that recognizes specific sequences of DNA. The position

of these sequences vary between strains. This generates different sets of fragments for different strains.

Despite their genetic heterogeneity particular CA-MRSA clones dominate in particular geographic regions. In the USA the USA300 clone (ST8) is dominant, while in Europe ST80, in the Austral-Pacific region the South-West Pacific clone (or ST30) and Queensland clone (or ST93) appear to predominate, in Taiwan ST59 and in Japan ST30 seem most common.

Panton-Valentine Leukocidin and USA300

Other important differences between isolates deemed HA- or CA-MRSA are that CA-MRSA are generally able to spread in the community, very often are Panton-Valentine leukocidin (PVL) positive, and until now are less resistant to non-ß-lactam antimicrobial agents. PVL is more common among CA-MRSA isolates than hospital-associated HA-MRSA and has therefore also been suggested to play a major role in the pathogenesis of skin and soft tissue infections. This, however, is only based on the association between PVL and skin and soft tissue infections. It should be noted that more than 90% of CA-MRSA in the USA are PVL-positive isolates in contrast to European isolates, where approximately half carry the genes encoding PVL. But at present nearly all US isolates belong to USA300 and are clonally related.

Panton-Valentine Leukocidin (PVL): PVL damages polymorphonuclear leukocytes and macrophages in vitro. Both important phagocytic cells of the immune system that can kill bacteria. PVL is strongly associated with CA-MRSA. It contributes to severe disease.

MRSA in animals

Humans are one of the most important hosts for S. aureus, but carriage and infections among animals are well-known, particularly among cows (bovine mastitis) and companion animals. Generally, carriage and infections are with MSSA, but in the last decade MRSA are also increasingly isolated from companion animals, milk cows, veal calves and especially pigs. MRSA from dogs, cats, and horses appear to be predominantly related to HA-MRSA and infections of milk cows (mastitis) have mainly been reported in Eastern Asia. The role of MRSA among companion animals and mastitis will not be studied due to the small number of isolates involved and their relationship with HA-MRSA.

Screening suggests that up to 40% of the Dutch pigs carry MRSA. Pig-related MRSA has also been demonstrated in France. All pig-related MRSA belong to ST398, or its evolutionary

descendants. However, two different SCCmec types, type IV and V, have been found within this ST. These isolates have also been found among approximately one third or half of all pig farmers and their household members. Furthermore, approximately a quarter of veterinarians in the pig sector are carriers of these MRSA lineages. Preliminary data indicate that this is not only the case for The Netherlands, but also for other EU member states. Although initially only observed among pigs, this strain is increasingly found in calves and also broilers. In addition, a case of endocarditis in humans caused by ST398 was described and it was isolated from a case of exudative epidermitis in pigs and also from dairy cattle suffering from mastitis. Although some microbiologists do not consider this strain to be a virulent pathogen, it poses nevertheless a major economic and health threat. For example, The Netherlands exports nearly a million tons of pig meat and 6 million live pigs. Approximately 10,000 companies are directly involved in this industry. In 2005 more than 5.5 million tons of pig meat were exported between EU member states. In addition, nearly 15 million live pigs (including MRSA-positive pigs), are transported across Europe each year. The most important other reservoir involved in export seem to be veal calves. In this intensive production system the calves (often male calves transported from other countries) receive large amounts of antimicrobials and it seems likely that this could be an attributing factor to the presence of MRSA. Export is vastly increasing the reservoir and the exposure of humans to this strain. In addition, it is well known that S. aureus virulence factors can be mobilized and easily exchanged between strains. This makes it only a matter of time before more virulent descendants of this strain will emerge. Currently, ST398 is almost exclusively found on farms, but further surveillance may find other STs as well. We designate MRSA carrying SCCmec IV and V obtained from farm animals, veterinarians and persons living on farms as LA-MRSA.

Problem definition

Using stringent infection control policies HA-MRSA was contained in several European countries and its epidemiological behavior is now predictable. In fact this behavior has been described in mathematical models. For CA-MRSA this is not the case. The emergence of CA-MRSA has all the characteristics of an epidemic with yet no possibilities for containment. LA-MRSA may even become a bigger problem considering the large animal reservoir. Community spread of CA-MRSA also implies that ß-lactam antibiotics, which are very successful in the community and also as prophylaxis in hospitals, can no longer be used. As a result patients have to be treated with vancomycin, which in all respects is a poorer antibiotic due to toxic effects, high costs, and mode of administration, amongst others.

Focus of CONCORD

The reason for the success of CA-MRSA is not understood. Knowledge about the strains and genetic mechanisms involved in the success is needed to develop adequate and cost-effective

counter-measures to control the emergence of CA-MRSA. In carrying out its research CONCORD will focus on CA-MRSA including LA-MRSA because:

- CA-MRSA is the most striking and alarming change in epidemiology of MRSA in the past 5 decades.
- Epidemiology of CA-MRSA is poorly understood in contrast to that of HA-MRSA.
- We are at the start of the epidemic. Containment still may be possible. In contrast to hospital MRSA (which seems more or less stabilized in most European countries).
- CA-MRSA is a major threat for existing control measures in hospitals (i.e. search and destroy policy).
- No effective control strategy for CA-MRSA exists.
- LA-MRSA is an economic problem.
- LA-MRSA is a resistance reservoir, e.g., for novel SCCmec variants.
- LA-MRSA may become a major human health problem as exemplified by MRSA carriage among farmers, veterinarians, and a human endocarditis case.
- LA-MRSA, especially in pigs is a natural model system to study epidemiology and effective counter measures.
- Acquisition of virulence factors (e.g., PVL) by LA-MRSA may result in a new highly epidemic and virulent CA-MRSA clone.
- The fully sequenced LA-MRSA strain, which was responsible for a case of human endocarditis provides a wealth of information on strain adaptation to the community and the human host.

Research objectives

To control and prevent infections by CA-MRSA, there is an urgent need to improve our understanding of the ecological success of CA-MRSA in the community in contrast to HA-MRSA and to develop counter-measures to control this community spread. This understanding is essential in order to develop effective strategies against CA-MRSA, for both human and farm-related MRSA (CA-MRSA and LA-MRSA, respectively). A distinction between CA-MRSA and LA-MRSA (and also CA-MSSA and LA-MSSA) is critical, because the reservoirs do not completely overlap and the farm-associated S. aureus strains may also show specific adaptations for their respective hosts.

1. To collect and characterize recent CA- and LA-MRSA from EU member countries in order to obtain a comprehensive picture of CA-MRSA in Europe.

2. To unravel the ecological success of MRSA outside the hospital by investigating genetic and transcriptional differences between HA-, CA- and LA-MRSA.

3. To determine the physiological role of gene products that contribute to the ecological success of CA-MRSA.

4. To develop mathematical modeling tools in order to quantify potential transmission control measures.

5. To develop effective intervention strategies for CA-MRSA and LA-MRSA.

Project Results:

LA-MRSA epidemiology

An important part of the project was the collection of relevant recent isolates and the collection of data concerning the transmission of MRSA.

The collection of isolates, data and characterization of LA-MRSA was the main focus of the work at DTU-FOOD. A total of 494 isolates were collected. Of these isolates 486 were MRSA isolates from pig farms which were isolated in ten member states (Belgium, Denmark, France, Germany, Hungary, Italy, Poland, Portugal, Spain, and the Netherlands). These isolates were previously isolated as part of The Baseline Studies in 2008, in the respective countries. Additionally, 87 MSSA isolates from pig origin were obtained from collaborators from four countries in Europe (Germany, Denmark, Ireland, and Poland) for comparison. Furthermore, farm data were obtained for mathematical modeling from France, Italy, Poland, Hungary, Ireland, Belgium, Portugal, and Denmark. Isolate data and 100 veal calf isolates were also collected from the Netherlands and data corresponding to 63 isolates (but not the isolates) was collected from Belgium. Additionally, we have obtained 100 isolates of MSSA isolated from veal calves in the Netherlands for comparison to the MRSA.

The LA-MRSA isolates recovered from pigs (486 isolates) presented spa types related in their great majority to CC398 (448 isolates), CC1 (21 isolates) and CC97 (14 isolates). CC5 and CC9 were also detected (one isolate each). The S. aureus collected from veal calves comprised 100 LA-MRSA which all had spa types related with CC398. Typing data obtained from Belgium also indicates that most spa types are related to CC398, and only one isolate has a non-related spa type. The LA-MSSA recovered from pigs (87 isolates) also had spa types that belonged mostly to CC398 (35 isolates), CC30 (19 isolates), CC9 (23 isolates), CC97 (two isolates), CC5 (two isolates), CC8 (one isolate), and CC1 (one isolate) were also detected. The MSSA from veal calves (100 isolates) also seems to present a higher degree of genetic diversity, including spa types related to CC398 (53 isolates), but also to CC133 (four isolates), CC97 (two isolates), CC151 (one isolate), CC1 (one isolate) and CC9 (one isolate).

SCCmec typing of 124 MRSA isolates including all the non-CC398 and a sample of the CC398 isolates revealed most frequently SCCmec type V cassettes which were identified in 84% of the MRSA from pig origin tested (94 out of 112). These included predominantly the SCCmec subtype Vc harboring the czrC gene encoding zinc resistance which was found in 69/112 isolates, but also other type V without this gene (25 isolates) Regarding the SCCmec type IV related subtypes (IVa and IVc) these were found in 16% (18/112) of the isolates from pig origin tested. Among the veal calve isolates the SCCmec type mostly detected was the type IV relates subtypes (IVa, IVc) which were present in 79% of the isolates tested whereas. Twenty one percent harbored a type V cassette. The same subtype could be found in MRSA originating from different countries and coagulase negative staphylococci retrieved from pig farms in Denmark. In the same way, additional typing data revealed similar types between LA-MRSA and CA-MRSA and methicillin-resistant coagulase-negative staphylococci of human origin.

Methicillin-resistant coagulase-negative staphylococci: S. aureus belongs to the larger genus of the staphylococci. These are divided into two groups based on their ability to coagulate plasma. S. aureus is coagulase-positive. Coagulase-negative staphylococci are part of normal (skin) flora of humans and live-stock.

Resistance to the antibiotics tetracycline and penicillin was found in all CC398 MRSA isolates tested. Resistances to the antibiotics trimethoprim, streptomycin, erythromycin, ciprofloxacin and tiamulin were also frequently observed among LA-MRSA.

Furthermore, we have also performed some studies aiming at the improvement of the detection of MRSA from animal samples. S. aureus is normally found on the skin and nares, surviving and growing under extreme conditions: dry environment with high salt and low pH (acidic conditions). In the selective isolation so far used high salt concentrations has been the main selection. We hypothesized that also pH adjustment could be used for selection of this species. In vitro studies indicated that lowering the pH would improve detection by reducing the background flora. This was also tested in real-life samples including nasal swabs and environmental dust samples for optimization of the methods. The results indicated that there was some improvement in detection with pH reduced to 5.5 in the pre-enrichment medium, for the nasal swab samples, however the results were not significantly different from the standard method and therefore more optimization might be needed in the future to improve detection.

Resistance towards metal compounds used in agriculture, was also a main focus of this project. First, it was observed that resistance to zinc was present in 74% of a strains collection of Danish CC398 isolates which showed significant association with the mecA status. A putative metal resistance gene was identified and cloned. Cloning of the gene czrC confirmed that it confers resistance to zinc chloride and cadmium acetate. This gene was detected within type V SCCmec elements newly classified as Vc and therefore genetically linked to the mecA gene.

In the strains collected within CONCORD zinc resistance was observed in 73% and 42% of MRSA CC398 from pigs and veal calves from Europe. By country, the prevalences of czrC varied from 34% to 91% among MRSA from pigs and 41% in the veal calf isolates. None of the MSSA tested carried the czrC gene. These results indicate that resistance to metals such as zinc and cadmium is widespread and may have played a role in co-selection of methicillin resistance in S. aureus CC398 in European countries.

CA-MRSA epidemiology

In order to obtain a comprehensive global perspective of the epidemiology, population structure and origin of community-associated S. aureus (CA-SA) in Europe, 569 MRSA and MSSA isolates collected from colonization and infection were recovered from the 16 most populous

European countries, from community and community-onset populations. Questionnaires addressing risk factors for previous hospital contact were filled for each isolate.

The genetic background of isolates was characterized by state-of-the-art molecular typing techniques (spa typing, pulsed-field gel electrophoresis and MLST) and the presence of virulence determinants usually associated to CA-MRSA isolates, namely PVL and the arginine catabolic mobile element, that both are associated with CA-MRSA, was assessed. Moreover the type of chromosomal cassette chromosome mec (SCCmec) carrying the determinant for methicillin resistance (mecA) was determined.

We found that 60% of all isolates were associated with epidemic community-associated genetic lineages. Most MRSA isolates belonging to these lineages were related with USA300 (ST8-IVa and variants) (40%), followed by the European clone (ST80-IVc and derivatives) (25%) and the Taiwan clone (ST59-IVa and related clonal types) (15%). A total of 52% of the MRSA carried PVL and 14% carried the arginine catabolic mobile element, which is assumed to contribute to virulence. Surprisingly, we found a high genetic diversity among MRSA clonal types (ST-SCCmec combination). Specifically, about half of the isolates carried novel associations between genetic background and SCCmec type, what contrasts sharply to what is observed in the United States, wherein a single clone is responsible for the great majority of infections in the community.

CA-MRSA clones: Different clones are prevalent in different geographic regions. In the USA USA300 is the dominant CA-MRSA clone, whereas in Europe this ST80 or the European clone; in the Far East it is ST59 or the Taiwan clone, whereas in the Austral-Pacific region the Queensland clone (ST93) and South-West Pacific clone (ST30) are dominant.

HA-MRSA clones: Also among HA-MRSA some clonal types are or were dominant. Often these showed pandemic spread. Examples are the Iberian clone, Brazilian clone, Berlin clone, New York/Japan clone, EMRSA-15, and 16.

Among the MSSA isolates associated to CA epidemic lineages, the majority belonged or was related to ST30 (27%), ST15 (17%), ST121 (11%) and ST7 (10%). The remaining clones were represented by less than 5% of the population.

Noteworthy, we found that a great part of the S. aureus isolates analyzed (27%), collected in the community and community onset populations belonged or were related to epidemic clones associated to hospitals, mainly to EMRSA-15 (ST22-SCCmec IVh), Berlin (ST45-SCCmec IV) and New York/Japan (ST5-SCCmec II) clones, suggesting the blurring of the boundaries between the hospital and community.

Analysis of isolates relatedness showed that MRSA and MSSA isolates belonging to ST8, ST72 and ST59 were highly related, suggesting that MRSA isolates belonging to these clonal types

may have emerged in Europe, whereas others like those belonging to ST30 and ST93 were probably imported from outside Europe.

Some asymmetry was observed in the number and distribution of clonal types found among countries. In Finland, Sweden and Poland, the most prevalent MRSA clonal type was related with the Taiwan clone (ST59-SCCmec V); in France and Greece the most prevalent clone was the European or related clones (ST80-SCCmec IVc); and in Bulgaria, Czech Republic, Denmark, Italy, Portugal, Romania, Slovakia and Spain the most prevalent was the USA300 or related clones.

In spite of the genetic diversity observed, all the clonal types identified were disseminated in more than one country and neighboring countries shared more clonal types than distant countries. The most epidemic clonal type among MRSA was the European clone (ST80-SCCmec IVc) that was found in ten different countries, followed by USA300 (ST8-SCCmec IVa) that was recovered in nine countries. Regarding MSSA, the most disseminated genetic background was ST15 and related clonal types that were identified in eleven different countries, followed by ST121 and ST30 and its derivatives that were identified in nine countries each.

Besides SCCmec other staphylococcal cassette chromosomes (SCC) have been described which could act as vehicles of transmission of virulence determinants and as players in SCCmec evolution. In order to assess the breadth of genetic diversity of SCC elements among MSSA and their role in SCCmec evolution we screened for the presence of cassette chromosome recombinases (encoded by the ccr genes) in CA-MSSA isolates from humans. Cassette chromosome recombinases excise SCC elements from the chromosome and integrate them into another chromosome after transfer to another strain). Our results showed a low frequency of SCC elements in CA-MSSA (7%), indicating that these isolates are not reservoirs of SCC elements. However, the few ccr gene variants found among MSSA isolates were already described or were related to those found in MRSA, suggesting that SCC elements from CA-MSSA may be involved in SCCmec evolution either by the acquisition or loss of mecA.

In order to understand the extent of dissemination of MRSA and MSSA strains among community and farms, the population structure of S. aureus collected from these two environments and characterized under the scope of this project was compared. Only a small proportion of types (6.27%, 14/223) were common to animals and humans, suggesting that the extent of dissemination between the two settings is not frequent. Also our results suggest that dissemination probably occurs in the two directions: from animals to humans and from humans to animals. Additionally, we observed that SCCmec is more diverse among humans than animals and that different animal species could be reservoirs of different SCCmec types: pigs for SCCmec V and calves for SCCmec IV.

Comparison of different MRSA sub-populations

When the grant proposal was prepared DNA sequence and transcription data for CA-MRSA were limited and non-existent for LA-MRSA. Our hypothesis for successful adaptation of a strain to a new environment supposed either the acquisition of novel properties or the differential expression of genes already present.

The occurrence of MRSA is often associated with outbreaks (e.g., EMRSA15/16). Recently, the emergence of livestock-associated (LA-MRSA) pathogens have complicated infection control. This change in epidemiological and microbiological characteristics will provide new challenges, as little information exists on the genetic determinants responsible for the epidemiology of CA- and LA-MRSA isolates. An array of typing methods have been used in epidemiological surveillance. MLST studies have shown that S. aureus has a highly clonal population structure, 87% of S. aureus in hospitals and the community belong to any one of 11 CCs. However, these methods only detect a limited amount of markers and many genetic changes remain unseen. Microarrays covering whole genomes and high-throughput sequencing devices are the two main techniques currently utilizable for whole-genome characterization. These tools not only provide information for the development of genotyping assays but also allow evaluation of potential virulence of the strains, by enumerating genetic-resistance markers and toxin content.

Microarrays: The genetic material DNA is made up of double-stranded DNA in which cytosine (C) matches with guanine (G) and thymine (T) with adenine (A). So, when two strains have matching sequences they can combine (hybridization). DNA encodes genes and regulatory sequences. Unique single-stranded pieces can be synthesized which match the different genes and regulatory sequences. These sequences are usually unique and called probes. The bacterial DNA is both fluorescently labeled and made single-stranded. This allows it to bind to the synthesized pieces (probes) and be recognized. The synthetic pieces of single-stranded DNA are spotted on a glass-slide and the position of each unique piece is known. This allows to determine whether certain genes and regulatory sequences are present or absent in an isolate. The slides with the pieces of synthetic DNA are called microarrays. Instead of chromosomal DNA RNA can be used to see which genes are expressed (transcriptome analysis.

Comparative genome hybridization (CGH): The use of microarrays to compare the gene content of different isolates.

However, microarrays used for epidemiological purposes suffered from several biases. To date, 120 sequenced S. aureus genomes are publicly available, 30 of these sequences are completed with annotation (i.e., the function of the genes is known). In this project, a new strategy for designing a nonredundant multistrain microarray based on this sequence information and on a tiled design on both strands of the DNA resulted in the most

comprehensive S. aureus microarray available today. The knowledge of the S. aureus genome we have so far, is shaped by the DNA sequencing of mainly human disease causing isolates. However, the population of S. aureus is not confined to humans, S. aureus is carried by, and can cause disease in animals as well. To design a microarray directed against the best possible representation of S. aureus genomes, sequence information of LA-MRSA strains was included in the design. Furthermore, a novel comparative genome hybridization (CGH) approach has been developed during the project. An algorithm is designed that makes use of the distribution of absent and present calls in a common reference to construct a mapping from signal to a statistical result. In this way for each probe a statistical result for presence is calculated for each strain. This approach resulted in an application for identifying the presence or absence of genes in S. aureus isolates at a whole genome and at a probe level that can be used for correlation studies. The correlation studies could lead to new insights of gene interactions within the pathogenicity of S. aureus.

This approach was used to detect differences in gene content from CA- and LA-MRSA in contrast to hospital-associated MRSA (HA-MRSA). Whole genome DNA analysis confirmed the existence of three different epidemical classes of MRSA as described earlier by gene based genotyping strategies. The three epidemical classes can grouped based on the SCCmec type. While CA-MRSA showed an overrepresentation of SCCmec IV, LA-MRSA isolates contained mainly SCCmec V subtypes, although subtype IV was found in one occasion. HA-MRSA isolates showed a specificity for SCCmec I. The SCCmec subtypes II and III were not found in this study. The classes also differed for the PVL genes (CA-MRSA specific), the formyl peptide receptor inhibiting protein (HA-MRSA specific) and the antibiotic resistance encoding genes catA, tetM, and qacA (encoding resistances to the antibiotics chloramphenicol and tetracycline and some disinfectants) (all present in HA-MRSA, but absent in CA-MRSA). Leucocidins, enterotoxins, and most proteases were absent in LA-MRSA. This seems to indicate that LA-MRSA do not have a survival strategy based on toxification. Another remarkable finding was all the tested LA-MRSA strains contained an ST398 type of superantigen-like proteins. It is not clear if these truncated versions are as effective as the superantigen-like proteins present in CA- and HA-MRSA. Functional studies should be performed to test this.

The formyl peptide receptor inhibiting protein: the formyl peptide receptor is important in recognizing bacterial invasion by the host immune system.

Superantigens: superantigens can non-specifically activate the immune system leading to overactivation.

Where genotyping of S. aureus isolates is almost a routine procedure, expression studies for S. aureus are scarce. This study compared gene expression of six CA-MRSA and one LA-MRSA isolate during culture in a synthetic medium named IMDM, IMDM supplemented with 5% CO2 and 1% human plasma, respectively. The assumption was that any consistent difference in

expression profiles in media with supplements as compared to IMDM might give an indication for specific regulation upon contact with that specific supplement. On the other hand, a difference between the isolates could imply different adaptation processes upon changing environments and might give an indication for the ability of causing various types of infection. Analysis of the trend of significantly up-or down regulated genes has shown a large variation in the initial response of the isolates over the media. Also, in all cases the amount of significant genes increased over time. This effect can be explained by the assumption that over time more secondary induced expression will occur. Moreover, a distinction can be made in the responsiveness of the isolates to the media.

First, discrimination can be made based on the type of supplement. The CA-MRSA strains contained two responsive classes that differed in the response to CO2 or human plasma. Apparently, one set of isolates interacted more intensive with CO2 related factors than with components of human plasma, while the reverse is true for the other set of isolates. An alternative infection pathway may underlie this difference. This was tested in a gene expression comparison between strains MSSA476 and MW2, two genetically almost similar CA-MRSA strains. The significant regulation of almost a quarter of the genes in a 3 hour time-frame in blood demonstrated once more that expression studies in culture medium alone are insufficient to explain the pathophysiology of infections. Furthermore, data obtained for one strain cannot be extrapolated to other strains even when they are closely related. A clear example are the iron metabolism related genes, immune evasion proteins, like staphylococcal superantigen-like proteins, proteases and toxins, like HlgABC, LukDE and SplABCD. For the two strains described here, the higher up-regulation of staphylococcal superantigen-like protein transcripts, encoding the staphylococcal superantigen-like proteins, in MSSA476 as well as the higher up-regulation of the genes for the toxins and proteases HlgABC, LukDE, SplABCD in MW2 might indicate a difference in survival strategy after phagocytosis. A hypothesis could be that MW2 is better able to lyse the polymorphonuclear leucocytes and thereby prevent killing, whereas MSSA476 could be able to evade the antimicrobial compounds produced by the polymorphonuclear leucocytes and thus able to survive within this cell. The difference between this two strains might reflect the two responsive classes. These data also show that we still do not understand regulation pathways in S. aureus and why they differ between closely related. Apparently these pathways differ and only when studied under relevant conditions we will be able to understand S. aureus pathophysiology including its role in adaptation to the community.

Second, the LA-MRSA isolate is not sensitive to any of the supplements. The supplements are based on interactions of Staphylococcus with human cells. The LA-MRSA strain might be adopted to pig specific conditions. The interaction of S. aureus ST398 with pig epithelial cells was analyzed in an ex vivo model. An expression study revealed that mainly metabolic processes were involved. Interestingly, from a pathogenicity island, called SaPIbov2, ehich was present in the genome only 2 genes were partly expressed. No virulence associated genes were included in the list of significantly regulated transcripts, although such genes are present in the genome. Either the trigger for expression of virulence associated genes is not present in any of the systems we used or the results indicate that no infection has taken place with pig epithelial cells. However, expression of genes involved in colonization could be detected. As

pigs do not seem to be affected by a S. aureus infection, they might serve as incubators that develop new virulent human directed S. aureus variants.

Last, to compare the expression of the seven isolates a common denominator was needed. We selected genes that were present in all isolates and called this collection the core genome. The core genome contains genes that are associated with central metabolism and housekeeping functions. Much to our surprise, transcripts for proteins encoded by the core genome show an isolate specific expression profile. Clustering of the core genome showed an initial separation between LA-MRSA and CA-MRSA isolates. Clustering indicates a close relation between ST5, ST30 and ST8 and ST80 than to ST59 and ST93. A connection between the relations and the responsiveness to the media could not be made. This once again showed that more in depth studies of gene expression in Staphylococcus are needed to understand S. aureus pathophysiology including its role in adaptation to the community.

MRSA adaptation through gene acquisition and mutation

Gene content and mutations

To test our hypothesis that small changes might lead to differences in regulation. We investigated three sets of isolates from patients who were first sampled as carrier and afterwards developed an infection with the same strain based on MLST and spa typing and a fourth set consisted of two ST398 isolates from a farmer and a carrier pig. Whole genome sequencing showed that one set of ST80 isolates showed several gene content and nucleotide changes (SNPs). It was concluded that these isolates are not a pair. The second set of isolates showed only one major difference (the presence of a phage). No SNPs were detected, but not all potential SNPs have been checked. The third pair from patients did not show any differences, but not all potential SNPs have been checked. For the two ST398 isolates no differences in gene content were observed. Twelve mutations were observed between the two isolates. No phenotype could be linked to the SNPs. However, a difference in the regulator MgrA (Multiple gene regulator A), that regulates several hundred genes, was noted between these isolates and the first sequenced ST398 isolate (S0385). A 6 nucleotide insert in S0385 leads to a lower expression of MgrA and increased biofilm formation, an important aspect of many S. aureus infections. Further analysis showed that the protein TraG encoded by the mobile Integrative Conjugative Elements present in LA-MRSA and some other strains also contribute to biofilm formation. Also ß-toxin which is usually intact in LA-S. aureus, but usually disrupted in human derived strains contributes to biofilm formation. Disruption of the ß-toxin gene led to lower colonization in a pig nasal explant model. ß-Toxin appears to play an important role in host adaptation.

CA- and HA-MRSA fitness

HA-MRSA is caused by only a few clonal lineages, whereas CA-MRSA is represented by a large number of different clonal lineages, although certain lineages predominate. In contrast LA-MRSA is mostly represented by a single clonal lineage, but this may be explained by host range adaptation of MRSA. The reason for the change in epidemiology is poorly understood. One of the aims of CONCORD is to explain the adaptation of CA-MRSA (including LA-MRSA) to the community. Most explanations focus on virulence factors or a burden due to the presence of SCCmec.

We hypothesized that HA-MRSA has adapted to the hospital environment due to antibiotic pressure, and that CA-MRSA results from wild-type MSSA that acquired mecA. Of the HA-MRSA isolates 67.5% had 5 rRNA operon copies compared to 23.2% of the CA-MRSA and 7.7% of MSSA isolates. In addition 105 MSSA isolates of cystic fibrosis patients were tested, because these patients are repeatedly treated with antibiotics during their lifetime. Indeed, 32.4% of these isolates had 5 rRNA operon copies. For all subsets a correlation between resistance profile and rRNA copy number was found. Next, we showed that in vitro antibiotic pressure may result in rRNA operon copy loss. We also showed with 51 isolates that S. aureus containing 6 rRNA copies are more fit than isolates with 5 copies, also after matching for genomic background. We conclude that HA-MRSA and cystic fibrosis isolates have successfully adapted to an environment with high antibiotic pressure by the loss of an rRNA operon copy. This loss has facilitated resistance development, which promoted survival in these niches strain fitness decreased, which explains their lack of success in the community. In contrast CA-MRSA retained 6 rRNA operon copies rendering them more fit and thereby able to survive and spread in the community.

rRNA operons: All bacterial cells have ribosomes to produce proteins. Ribosomes consist of protein and 3 ribosomal RNA (rRNA) molecules. The genes encoding these rRNA molecules are next to each other on the bacterial chromosome and transcribed as a single unit (an operon). The number of rRNA operons is different for each species. For S. aureus 6 copies is believed to be optimal. For other species this can be a different number.

<u>CA-and LA-MRSA virulence</u>

Ex vivo pig nose mucosa explant model

To study the interaction of ST398 in pig nose tissue, Utrecht University developed an ex vivo pig nose mucosa explant model, that also could be used for intervention studies. This model consisted of porcine nasal mucosa explants cultured at an air-liquid interface. In cultured mucosa explants from the surfaces of the ventral turbinates and septum of the pig nose no changes in cell morphology and viability were observed up to 72 hours of culturing. MRSA colonization on the explants was followed with three MRSA ST398 isolates for 180 minutes. The

explants were inoculated at 3x108 colony forming units/mL and a decline in the number of colony forming units was observed for all MRSA strains during the first 30 minutes. Subsequently, the isolates showed either bacterial growth, no growth, or a further reduction in bacterial numbers. The MRSA bacteria were either localized as clusters between the cilia or as single bacteria on the cilia surface. No morphological changes in the epithelial layer were observed during the incubation with MRSA. It was concluded that porcine nasal mucosa explants are a valuable ex vivo model to unravel the interaction of MRSA with nose tissue.

To investigate which genes are expressed during pig nose colonization, the expression of genes of MRSA ST398 during colonization in the ex vivo model was studied. MRSA bacteria were isolated at different time points and RNA was isolated. Transcription profiles were obtained with the microarray described above. A number of genes were identified that are up or down regulated, and experiments to establish their role in the successful spread of MRSA ST398 are in progress.

Gene regulation in CA-MRSA

Where genotyping of S. aureus isolates is almost a routine procedure, expression studies for S. aureus are scarce. This study compared gene expression of six CA-MRSA and one LA-MRSA isolate during culture in IMDM, IMDM supplemented with 5% CO2 and 1 % human plasma, respectively. The assumption was that any consistent difference in expression profiles in media with supplements as compared to IMDM might give an indication for specific regulation upon contact with that specific supplement. On the other hand, a difference between the isolates could imply different adaptation processes upon changing environments and might give an indication for the ability of causing various types of infection. Analysis of the trend of significantly up-or down regulated genes has shown a large variation in the initial response of the isolates over the media. Also, in all cases the amount of significant genes increased over time. This effect can be explained by the assumption that over time more secondary induced expression will occur. Moreover, a distinction can be made in the responsiveness of the isolates to the media as discussed above for MW2 and MSSA476.

To survive under changing environments, a microorganism needs to be able to quickly adapt gene expression. In the last decade, the influential role of small RNAs (sRNAs) in bacterial gene regulation has been demonstrated for a diverse array of genes and various prokaryotes. In S. aureus, hundreds of intergenic regions have been identified that could encode a sRNA candidate. However, the functional role in gene regulation is still largely unknown. More comprehensive knowledge of regulatory RNAs will be essential to fully understand staphylococcal colonization and pathogenicity.

sRNA: sRNAs are small RNA molecules (on average at least 10-fold shorter than protein encoding RNAs) that play a role in the regulation of gene expression by binding to protein encoding RNAs there by either enhancing or reducing their translation into protein depending on the type of sRNA.

Here, we have used transcriptome data acquired from microarray analysis on five independent highly-reproducible growth curves in synthetic medium to predict sRNA candidates in the intergenic regions of S. aureus. A total of 115 putative sRNAs were identified. The computer program IntaRNA was used to predict potential mRNA targets involved in virulence for these putative sRNAs. Five sRNA candidates remained for further characterization. In vivo analysis of two strains where sRNA Msa079 and Msa004 were knocked-out in MSSA476, showed a trend in post-transcriptional regulation of the predicted targets, extracellular fibrinogen binding protein (Efb) and delta-toxin (Hld). However, in vitro hybridization assays to confirm binding of the sRNA and the protein encoding mRNA, did not confirm the in vivo trends of regulation. The in vitro sRNA/mRNA interactions were weaker than was expected from the in vivo data. The results of the sRNA regulation experiments described here show the difficulties of studying sRNA regulation and may contribute to better understanding virulence gene regulation via sRNAs in S. aureus.

SCCmec reservoir

Another question that was addressed in the project was if coagulase-negative staphylococci in pig farms could be a reservoir for SCCmec transfer to S. aureus. MRSA likely originated by acquisition of the SCCmec from coagulase-negative staphylococci and the presence of SCCmec elements in pig farms was investigated. It was unknown whether the same SCCmec types are present in MRSA and coagulase-negative staphylococci that reside in the same niche. We identified on 10 pig farms the presence of different coagulase-negative staphylococci species that harbored heterogeneous SCCmec elements. All S. aureus belonged to ST398, with SCCmec types V and IVa. Type IVc as well as type III, VI and novel subtypes of type IV and not-typeable types were found in coagulase-negative staphylococci. S. aureus, S. epidermidis and S. haemolyticus shared SCCmec type V. Noteworthy is the presence of SCCmec type IVc in several staphylococcal species isolated from one pig farm that suggests exchange of this SCCmec type in coagulase-negative staphylococci, but the general distribution of this SCCmec type still has to be established. We conclude that staphylococci on pig farms act as a reservoir of heterogeneous SCCmec elements, and that these staphylococci may act as source for transfer of SCCmec to S. aureus.

<u>Mathematical modeling of MRSA transmission</u>

Introduction

Two novel mathematical models to study MRSA transmission in different settings were developed. A LA-MRSA between-farm transmission model and a between-hospital transmission model of health care associated infections. In our analyses we also employed an existing genotype-specific within-hospital pathogen transmission model. The models are flexible,

extensible, and reusable beyond CONCORD activities: as long as basic assumptions are verified and there exist suitable data sets, they can be employed to perform, in other countries, similar analyses of MRSA transmission potential in hospitals, health-care networks, or different livestock industries.

LA-MRSA in the Danish pig industry

Movement-induced transmission alone can yield a high probability of LA-MRSA persistence at very low prevalences. The discrepancy with simple epidemic models, in which lower probability of persistence is associated with lower prevalence, arises from the strong fragmentation of the pig-trade network. Due to the high volume and frequency of between-farm trade, LA-MRSA eradication may be difficult. There are currently no practical, low-cost, farm-based control strategies that will effectively tackle endemicity in the pig industry.

Endemic prevalence levels obtained with 2008 movements (1.8% [90% CI 0.08-2.27%]) are comparable with prevalence estimates from the European Food Safety Authority baseline survey (2.4%). However, our model is not able to reproduce the results of more recent surveys in years 2009 and 2010, which found 12.7% and 16.2% of Danish pig-farms affected by MRSA, respectively. This discrepancy could arise from year-to-year variation in the movement network, from additional

transmission mechanisms, or from multiple introductions (including multiple episodes where CC398 MSSA may have become resistant).

CI: CI stands for confidence interval a statistical term that indicates how reliable a result is. A larger confidence interval indicates that the result is less certain.

Health care associated infections in the Dutch and English health-care networks

The referrals of patients play an important role in the spread of nosocomial infections. Each index hospital in England (the Netherlands between brackets) can affect, on average, up to a maximum of 106 (56) other hospitals, which correspond to 73% (57%) of the health-care network. Employing an empirical approach we have built a hospital priority list to aid in the selection of surveillance sentinels. Our model supports the traditional approach in the design of surveillance programs, that preferentially targets the most connected elements in a network. With this selection scheme, employing 20% of hospitals as sentinels can be a relatively effective surveillance method when there is limited availability of resources, as detection occurs 70% earlier than with surveillance restricted to the best sentinel hospital. In the endemic regime that arises when implementing complete pathogen elimination from individual hospitals, high complexity hospitals re-acquire the nosocomial pathogen 4-6 times faster than low complexity ones. Consequently, these hospitals must implement control measures more frequently in order

to remain free from the health care associated infections, increasing the costs associated with the maintenance of a pathogen free environment.

Genotype-specific within-hospital transmission potential of MRSA

In Dutch hospitals, MRSA ST398 is 5.90 times less transmissible than non-ST398 (95% CI 2.24-23.81) and in Danish hospitals, CA-MRSA is 9.3 times less transmissible than HA-MRSA. The following estimates for the basic (R0) and single admission (RA) reproduction ratios were obtained (for an explanation of R0 and RA see next page):

R0: R0 is the basic reproduction rate is the average number of secondary infections/colonizations produced when an infected/colonized individual is introduced in a completely susceptible host population. When R0 is above 1 the bacterium will spread throughout the population. When R0 is smaller than 1 it will die out.

RA:RA is the single admission basic reproduction rate. This is the average number of secondary infections/colonizations produced when an infected/colonized patient is introduced in a completely susceptible hospital population during a single admission.

R0 and RA estimates include 90% and 95% CIs, and were obtained with the between-farm, and genotype-specific within-hospital models, respectively. R0 corresponds to pig movement-mediated transmission, and the value shown was obtained with 2008 movement data. RA in Dutch hospitals was calculated with data from 55 hospitals.

Pig decontamination

On farms, using bacteriophages for biological decontamination is a potential approach which has not previously been fully exploited. The selection of appropriate phages for phage therapy of LA-MRSA was performed by Novolytics. A combination of two lytic Staphylococci phages, demonstrated good infection coverage against pig and ST398 MRSA strains. Between them, they infected 90% (17/19) of the tested pig and ST398 MRSA strains. A novel phage was also isolated from a pig nose swab, this phage appeared to specifically target pig and ST398 MRSA strains and was very stable at both 4oC and room temperature. The novel phage demonstrated great potential for veterinary therapeutic usage. The in vivo activity of phages against MRSA was studied in a piglet model aiming at decolonization of the pig nose. Additionally an ex vivo model using explants of pig nose mucosa was studied for the phage activity against MRSA (The data of both the in vivo and ex vivo experiment are confidential). In conclusion, the data obtained and the models developed in the project are critical advances towards the

development of a phage treatment for the eradication and treatment of MRSA, both in livestock and humans.

Potential Impact:

General

CONCORD is directly aimed at the topic: HEALTH-2007-2.3.1-4: Molecular epidemiology to control nosocomial and community spreading of highly virulent multi-drug resistant strains of bacterial pathogens. Following the execution of its work plan generated the following impact.

Increased knowledge about antibiotic resistance by explaining the epidemiology of a highly virulent and multi-drug resistant pathogen that shows the most striking and alarming change in epidemiology of S. aureus in the past decades.

In order to prevent the emergence of new strains of CA-MRSA and to develop effective and rapid tools to contain the present threat there was an urgent need to collect and characterize CA-MRSA from different reservoirs (e.g. humans, pigs, other) from EU member countries and to unravel the mechanisms explaining the ecological success of CA-MRSA.

CONCORD collected and characterized recent CA- en LA-MRSA from EU member countries and obtained a comprehensive picture of CA- and LA-MRSA in Europe. The isolates were characterized for several epidemiologically relevant characteristics including MLST, spa-type, SCCmec. This led to a representative collection of CA-MRSA, LA-MRSA, and CA-MSSA for future reference and study. The characterization data revealed the population structure of the European CA-MRSA and its relationship with the complete S. aureus population structure. This provided insight in the emergence and evolution of both CA- and LA-MRSA and showed which strains are best adapted to the community.

In addition factors required by MRSA for adaptation to become successful CA- or LA-MRSA were identified in a contemporary collection of a diverse group of MRSA. This resulted in an assessment of gene content and gene expression differences between HA-MRSA, CA-MRSA, CA-MSSA, and LA-MRSA and a more complete catalogue of virulence genes, phages and plasmids that will be used to study the distribution of virulence genes in the CA- and LA-MRSA population and the epidemiology of mobile genetic elements.

Another objective was to study acquisition of virulence factors such as PVL in other reservoirs (e.g. pigs), in order to contain possible CA-MRSA outbreaks in the earliest stage of disease development. There are potentially numerous mechanisms - or virulence factors that could contribute to CA- and LA-MRSA strains causing infections in humans and animals. CONCORD elucidated mechanisms that contribute to the potential success of CA-MRSA strains, including PVL, which aids in the destruction of white blood cells. Moreover, the presence of a gene - or its transcription - are not necessarily sufficient for the physiology of successful CA-MRSA. Expression of proteins under relevant conditions and their function ultimately determine the outcome if a strain is successful in the community. Hence determination of the expression of proteins was important.

Humans are one of the most important hosts for S. aureus, but carriage and infections among animals are well-known. Taking in to account that S. aureus virulence factors are present on

mobile genetic elements that can be easily exchanged between strains, it is highly likely that gene transfer between different reservoirs also plays an important role in the ecology of CA-and LA-MRSA. In order to obtain an understanding at the molecular level of the physiological requirements for successful adaptation of MRSA to the community and to develop effective transmission control measures CONCORD focused on both human and animal reservoirs.

The research was translated by means of the testing of novel measures for the containment of CA- and LA-MRSA. If the number of infections with CA-MRSA isolates increases significantly, physicians may need to change their treatment of presumptive S. aureus. Community spread of CA- and LA-MRSA implies that ß-lactam antibiotics, which are very successful in the community and also as prophylaxis in hospitals, can no longer be used. As a result patients have to be treated with vancomycin, which in all respects is a poorer drug. Although novel classes of antibiotics might offer durable solutions to treat infections in the long run, more seems to be needed if we are to prevent CA-MRSA from getting pandemic. Control can be achieved by using two approaches: reduction of the current levels of CA-and LA-MRSA and prevention of CA- and LA-MRSA spread.

In order to reduce current levels of CA- and LA-MRSA CONCORD focused on demonstrating a role for phage therapy. As the development of antibiotic resistance in pathogenic organisms is becoming a major global problem as several pathogens have even started developing resistance to the two new antibiotics introduced in the last couple of years and there are very few new antibiotics in the pipeline of the pharmaceutical companies there is an urgent need for viable alternative for the treatment and control of antibiotic-resistant bacteria. One of the more promising alternatives seems to be provided by phage therapy. Within CONCORD we aimed to demonstrate the effectiveness of phage therapy in pigs. For this we have included the services of Novolytics. Novolytics is currently developing an aqueous suspension to treat nasal carriage of MRSA. The potential use of phages in the development of novel and unique therapies for disease-causing bacteria is virtually limitless.

A very important feature of phage therapy is that bacteriophages do not infect human or animal cells.

Phage therapy is most likely also an option for treatment of pigs and humans. Prevention however is a more long-term solution. The best way to achieve prevention is by vaccination. CONCORD identified vaccine candidates that can/will be further developed in a subsequent project as the vaccine candidates could not be completely tested within the time frame of this grant, but the potential of these candidates was demonstrated in this approach.

Vaccination approaches to control S. aureus and thereby MRSA have been described in literature but their results are disappointing. We came to the conclusion that vaccines should be directed to different subpopulations A similar approach has been followed for pneumococci and meningococci, where only the most important serotypes/serogroups are included in the vaccine. To achieve this goal whole genome sequence data of at least the dominant European clones was analysed in silico for virulence factors.

A number of steps were needed for bringing about the impacts. Summarised the following results were be achieved by CONCORD:

- Obtaining a complete picture of CA- and LA-MRSA in Europe;
- Explaining mechanisms for successful adaptation of strains to the community;
- Definition of the role of virulence gene products;
- Development of transmission models;
- Development of interventions by (a) demonstrating the potential use of phage therapy in pigs and (b) identifying possible vaccine candidates.

LA-MRSA

The potential impact of the project for LA-MRSA can be summarized in the following points and described more in more detail below:

- Increased knowledge of LA-MRSA clones in pigs and veal calves in Europe and their dissemination among animals in European Countries.
- Construction of a strain collection and data collection which could be used within the project and which can be available for future studies.
- Contribute to a better understanding on the relationships between LA-MRSA as potential reservoir for CA-MRSA.
- Contribute to the knowledge about potential factors implied in the emergence and spread of MRSA in livestock and potential targets for interventions.
- Contribute to the scientific experience in the field of young scientists involved.
- Contribute to improve the capacity of researchers for advising policy makers regarding MRSA in Europe.
- Contribute to the knowledge that coagulase-negative staphylococci on farms are a potential source of SCCmec.

The characterization of the isolates from animal origin and comparison to the CA-MRSA from humans showed a more clear picture of the epidemiology of MRSA clones in Europe. As in other studies, the characterization performed in animal strains revealed that CC398 was the main clonal complex which is found widespread in Europe among livestock, contributing to more than 90% of the LA-MRSA. However, other CC were also found, such as CC1, CC5, CC8, CC9,

CC30 and CC97 which have been found in some of the countries in Europe and elsewhere. Furthermore, it is also known that other CCs are predominant in other parts of the world such as in Asia where it is CC9 that is mostly found among livestock

MRSA of animal origin have been discovered only in recent years but they were found widespread in pigs, veal calves and other domestic animal species around the world. Several factors can have promoted the acquisition of SCCmec cassettes by livestock adapted CC398 and the selection of these MRSA, but none of them fully explains, so far, how these bacteria became so successfully maintained in the farms and spread and evolved to what is observed today. Factors like antimicrobial use, tetracycline, and zinc have been hypothesized as potential contributors for selection of LA-MRSA. Data was collected for a better understanding of spread within farms and between farms under the scope of mathematical modeling within CONCORD. Also it was observed that the SCCmec elements involved show unexpected variability, and besides the most common V and IV subtypes found in this study, others have been described, including type III, VII-like, IX and X which were found in LA-MRSA isolated from animal origin around the world. This variability between the SCCmec types and subtypes that can be found among LA-MRSA indicate that SCCmec elements have been acquired several times in the past and recombination occurs frequently, representing challenges for the typing as well as to predict the future evolution of LA-MRSA. It is also noteworthy to recall that similar SCCmec elements were found in MRSA of human and animal origin, but also among coagulase negative staphylococci, which might act as reservoirs for the SCCmec elements. Furthermore, recent studies by others indicate that MRSA of CC398 has most likely evolved from human related MSSA which have been transferred to the animal population where they then acquired the tetracycline and methicillin resistances and evolved further as LA-MRSA. This recent knowledge gives a different perspective to MRSA and the way it might adapt to different hosts and a broader view of the issues associated with LA-MRSA emergence and spread.

The studies performed on LA-MRSA within CONCORD where also very timely, as this is a major public health concern at the moment for all players involved in issues related with antimicrobial resistance in animals. In the near future, there will probably be further concerted measures taken to continue following up on this issue, both at the National levels, but also at international level, including the focus of the European Commission which will be taking measures at European level.

The consortium has provided scientific support and results which will be very useful for the policy makers, researchers, clinicians and patients around the world, but also to general society, by generating more information on this subject. Furthermore, the partners in CONCORD gained in experience in their fields, but also in interchanging experiences with the other research groups and other related research areas and have participated actively in disseminating the results through their networks and through other media. Furthermore, in the role as providers of advisory service to national and International bodies DTU-FOOD has used both scientific results and knowledge obtained within CONCORD for the response to questions posed by policy makers and also for the design of studies. Furthermore, elements of the research group at DTU-

FOOD and collaborators have been invited to take part in a European Food Safety Authority Workgroup preparing recommendations of future monitoring of LA-MRSA at European level.

Characterization of methicillin-resistant coagulase-negative Staphylococci on pigs provided new information for the society. Awareness that on pig farms not only MRSA ST398 are present but also methicillin-resistant coagulase-negative staphylococococci, that are a potential reservoir for transfer of SCCmec elements to S. aureus is important. However, what the potential frequency of SCCmec transfer to S. aureus needs to be established.

CA-MRSA

The potential impact of the project for CA-MRSA can be summarized in the following points:

- A tremendously high level of genetic diversity among CA-MRSA from humans in Europe was discovered impacting on diagnostics, surveillance and control.

-A close watch of exchange of MRSA between different reservoirs is required.

In this study we identified a tremendously high level of genetic diversity among CA-MRSA from humans in Europe as well as a high frequency of PVL-positive isolates. This scenario poses an unprecedented challenge not only to diagnostics but also to infection control. The rapid CA-MRSA evolution in Europe demands a continuous surveillance as a means to help local health-care providers in designing strategies to detect and control CA-MRSA.

Also we observed that presently the extent of dissemination between humans and animals is not clinically significant, but animals continue to constitute an important reservoir of antimicrobial resistance determinants. Consequently, close watch of transmission between the two environments should be constantly carried out.

Genome analysis

-Contribute to the knowledge of the S. aureus population structure by defining subpopulation that impact on vaccine development.

-Contribute novel genes that define host specificity.

The hypothesis that differences in gene content might explain the difference between HA-MRSA and CA-or LA-MRSA was evaluated by comparing the genome content of 48 isolates. Isolates were classified as HA-MSSA (2), HA-MRSA (17), CA-MRSA (11), LA-MRSA (6) and sporadic isolates (12). Comparative genomic hybridization analysis not only showed that a

difference in gene content exist between the classes but also indicated that sporadic isolates could be assigned to either a HA-MRSA or CA-MRSA class. The sporadic clones could be evolutionary intermediates within one of the epidemiological classes of S. aureus. Remarkably, we did not find such an intermediate for LA-MRSA isolates. However, within the LA-MRSA isolates a clone was found that could be typed as SCCmec IV instead of SCCmec V. SCCmec IV is overrepresented within CA-MRSA isolates. So, although the epidemiological classes are distinct from each other, intermediate clones are found. It is this gene flow indicated by intermediate clones, that is of concern to society as this could lead to S. aureus variants that are highly virulent. The intermediate forms are dependent on a host to survive. This host could be human or farm-associated. Farm-associated hosts can be viewed as silent incubators to S. aureus isolates that eventually develop characteristics that makes them highly infectious to humans. Although the first isolation of an LA-MRSA strains comes from a patient that suffered from endocarditis, this study indicated that LA-MRSA isolates are not very virulent: they colonize pigs but do not infect them. They also lack some antibiotics resistance encoded genes. To avoid resistance to these genes, use of antibiotics at farms should be discouraged. A strict regime of hygiene without the aid of antibiotics could prevent colonization of pigs. This regime should be checked by a regular control of pigs by a nose swap. SCCmec typing and comparative genomic hybridization analysis of the DNA content could be used to monitor the characteristics of the isolate. This approach could initially be used to investigate to which extent farmers, their family and veterinarians are infected via LA-MRSA variants. This should give an answer to the question if a human intermediate is needed to turn the LA-MRSA isolate into a virulent isolate. It should also give a clue about the frequency of the transition from pig to human and from the primary infected human to other humans. As the infection of pigs occurs mainly in the nose, an approach based on bacteriophages directed to LA-MRSA strains might serve as an alternative. In this way, variants are killed before they develop to a virulent form.

However, such an approach is not possible if the intermediates develop in humans. A specific feature of CA-MRSA is the presence of the PVL. The presence of PVL can be detected at both DNA and protein level. It is advisable to test the presence of this protein or the encoding gene as part of the initial S. aureus screening to monitor new variants entering the hospital. New variants can develop by mutation of existing genes or by horizontal transfer of external DNA. Sequencing of new CA-MRSA clones could reveal the gene source causing the rise of new variants.

Several genes such as von Willebrand factor binding protein, ß-toxin play an important role in host specificity. First by contributing to immune evasion and the second to biofilm formation. In addition, adaptation of a MgrA variant in LA-MRSA was shown to contribute to biofilm formation. The isolate was obtained from a patient with endocarditis. The MgrA variant helps to explain the pathogenesis of this infection and the important of MgrA in biofilm formation.

Virulence

The potential impact of the project for MRSA virulence can be summarized in the following points:

-The establishment of different MRSA subpopulations with different virulence genes, e.g., toxins and immune evasion molecules will influence vaccine design.

-The discovery that TraG also contributes to biofilm formation will offer a new/additional target for the prevention of biofilm formation.

-Knowledge that gene regulation in one strain is not predictive for closely related strains will influence the evaluation of gene expression for vaccine development.

-The discovery that sRNA also can regulate virulence genes will influence our thinking how MRSA can adapt to new ecological niches.

-The finding that HA-MRSA adapted to the hospital will influence our thinking of MRSA evolution, but also that of other antibiotic-resistant pathogens.

-PVL

-PVL receptor

-The ex vivo pig nasal explant model will be used to study colonization and decontamination measures.

The clear delineation of different S. aureus populations based on comparative genomic hybridization shows that these subpopulations have different gene content. Clearly, LA-MRSA lack moist toxins and have a different set of immune evasion factors. These data combined with the data concerning PVL that many S. aureus virulence factors are host specific. As a consequence vaccine studies should be performed in more appropriate models. Mice are clearly not a good model for vaccination. This explains at least part of the failures for S. aureus vaccine development. The data concerning the differences in expression between closely related isolates show that it is imperative to know which genes/virulence factors are expressed to understand pathogenesis of the infection as well as design optimal vaccines. Together this has led to a new paradigm for S. aureus vaccine development. S. aureus vaccines should be subpopulation based; include virulence factors that are expressed at the site of infection; include a mix of antigens including immune evasion molecules; be tested in an appropriate animal model. A grant proposal based on this paradigm is currently being prepared.

The discovery that HA-MRSA adapted to the hospitals more than CA-MRSA to the community overturns an old dogma that CA-MRSA adapted to the community as a new ecological niche. This insight will alter our view of adaptation of bacterial species to antibiotics.

The ex vivo nasal explants model allows the use a more realistic model than culture cell lines for the study of nasal colonization and decontamination. Cell line are monocultures of often immortalized cells, whereas the model system maintains the variety of cells present in the nasal epithelium as well as its structure. Important is that in porcine nasal mucosa explants the bacterial and host factors can be evaluated under controlled conditions. First data have been obtained that MRSA strains may differ in the persistence in porcine nasal mucosa and indicated that ST398 MRSA strains were in general good colonizers. Further characterization of the strains differences is required to establish if nose colonization can explain the successful spread of ST398

Mathematical modeling

The potential impact of the project for MRSA virulence can be summarized in the following points:

-The models can be reused or combined with other tools for future studies.

-The studies may impact on the design of control strategies.

A common feature of these models is their reusability beyond CONCORD activities: as long as basic assumptions are verified and there exist suitable data sets, they can be employed to perform, in other countries, similar analyses on MRSA transmission potential in hospitals, health-care networks, or different livestock industries. The models can also be combined with other mathematical tools to better test control strategies and their cost-effectiveness. An example is the possibility of merging the between-farm transmission model with a farm-to-human model focused on the spill-over of LA-MRSA from a farrow-to-finish farm to the surrounding community, developed by members of the FP7 PILGRIM consortium.

In addition, two results may have some impact in the design of future control strategies.

1) Results obtained with the between-farm model show that because of the high volume and frequency of between-farm trade, there are currently no practical, low-cost, farm-based control strategies that will effectively tackle endemicity in the pig-industry.

2) Results obtained with the between-hospital model show that infection control strategies must at least have a regional scope, and control measures may have to be targeted to individual hospitals to effectively tackle the spread of health care-associated infections. In particular, resource allocation to large, complex hospitals will be needed for both early detection of novel pathogens, and their elimination from the health-care network after successful spread.

Pig decontamination

Insight in the feasibility of phage treatment of pigs was obtained that will impact on the future decolonization of animals and humans.

Antibiotic resistant bacteria (particularly MRSA) place a serious and increasing burden on society. They are responsible for increasing human and animal morbidity and mortality and the resultant social and economic costs of dealing with these infections is increasing The increase in the prevalence of antibiotic resistant pathogens combined with the limited number of new antibiotics being developed by pharmaceutical companies means that there is an urgent need for novel alternatives for treatment and control of antibiotic resistant bacteria. One potential treatment is the use of bacteriophages. The work undertaken during this project set out to determine the efficacy of phages in the treatment of MRSA colonized piglets as a potential method for controlling LA-MRSA. The data obtained and the models developed in the project are critical advances towards the development of a phage treatment for the eradication and treatment of MRSA, both in livestock and humans. At this moment no suitable method for application of phage treatment for decontamination of MRSA pigs or pig farms is available. Research into the activity of phages in vivo and the delivery method for decontamination of surfaces is ongoing.

Reducing MRSA colonization in farms could help reduce its spread through the environment and to humans, greatly reducing the burden these infections currently place on society. Phage treatment was studied as a way to reduce the carriage and spread of MRSA in pig farms. Data on the in vivo activity of phages against MRSA in colonized piglets was obtained, but how active the phages are in vivo and how accessible the MRSA bacteria are, are complex issues, which are still being addressed. During this project, a novel MRSA ST398 targeting phage was isolated in this project by Novolytics, and they are working on the further development of the phages and the phage delivery method. Although a final phage therapy approach for the decolonization of pigs has not been delivered in this project, valuable knowledge has been gained, that will enable future development of the phage therapy for the decolonization of farms which could significantly reduce the burden this disease currently places on society.

List of Websites:

Website

The project has made data and references to publications made available on a website:

www.concord-mrsa.eu

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