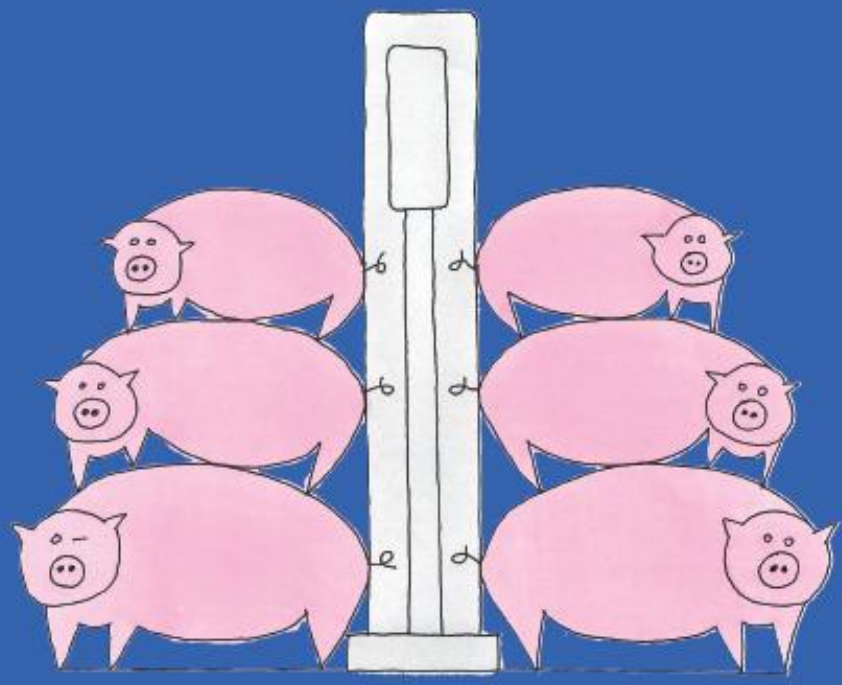
Epidemiology of Livestock-associated Methicillin resistant *Staphylococcus aureus*



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Epidemiology of Livestock-associated Methicillin resistant *Staphylococus aureus*

An academic assay in Medical Science

Doctoral thesis to obtain the degree of doctor from Radboud University Nijmegen on the authority of the Rector Magnificus, prof. dr. S.C.J.J. Kortmann, according to the decision of the Council of Deans to be defended in public on Thursday March 22, 2012, at 15.30 hours

by

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Index

List of abbreviations

BDGO	BD GeneOhm assay
BI	betrouwbaarheids interval
Bp	base pare
CAI	community acquired infection
CA-MRSA	community associated methicillin resistant *Staphylococcus aureus*
CC	clonal complex
ccr	creedence clearwater revival
CFU	colony forming units
CI	confidence interval
CLSI	Clinical Laboratory and Standards Institute
DNA	deoxyribonucleic acid
EARSS	European Antimicrobial Resistance Surveillance System
EMRSA	epidemic methicillin resistant *Staphylococcus aureus*
HAI	hospital acquired infection
HA-MRSA	hospital associated methicillin resistant *Staphylococcus aureus*
HCW	health care worker
LA-MRSA	livestock associated methicillin resistant *Staphylococcus aureus*
mL	milli liter
MLST	multilocus sequence typing
MRSA	Methicillin resistant *Staphylococcus aureus*
NT-MRSA	Non-typable methicillin resistant *Staphylococcus aureus*
OR	Odds ratio
ORF	open reading frame

PBP	penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
RAPD	random amplified polymorphic DNA analysis
PVL	Panton-Valentine Leukocidin gene
RIVM	Rijksinstituut voor Volksgezondheid en Milieuhygiëne
SCC*mec*	Staphylococcal cassette chromosome *mec*
SNP	single nucleotide polymorphism
Spa	Staphylococcus protein A gene
ST	sequence type
UTI	urinary tract infection
VRE	vancomycin resistant enterococci
WIP	Werkgroep Infectiepreventie

Chapter 1:Introduction:

Methicillin resistant *Staphylococcus aureus* in humans – past and present

Methicillin resistant *Staphylococcus. aureus* in animals

Methicillin resistant *Staphylococcus aureus* in humans – past and present

Staphylococcus aureus is an important pathogen in humans and the emergence of methicillin resistance is a hallmark in the history of resistant micro-organisms that have arisen since the advent of antibiotics. Prior to the discovery of antibiotics, *S. aureus* sepsis resulted in the death of more than 80% of patients, but this changed radically with the introduction of penicillin in the 1940s. Unfortunately *S. aureus* soon acquired beta-lactamase genes and in the early 1950's the predominant hospital *S. aureus* clones (phage type 80/81) had acquired resistance to penicillin, streptomycin, chloramphenicol, tetracyclines, erythromycin, novobiocin and kanamycin soon after they were introduced. When the beta-lactamase resistant antibiotic, methicillin, was then developed, it was said that "*A penicillin resistant to staphylococcal penicillinase (may) finally silence the adaptable staphylococcus*" [1]. Within a year after its introduction, the first methicillin resistant isolates were found in a London laboratory but at that time they were thought to be of little clinical significance. In 1964 Kirby still stated that *"initial studies five years ago led some of us to predict that the emergence of methicillin resistant staphylococci would not appear to become a problem. This … now appears to be correct"* [2].

But in the 1960's MRSA outbreaks were reported as far apart as Australia, Uganda and Denmark. In the latter, the prevalence of MRSA in blood cultures increased from 10% in 1966 to 46% in 1970 [3]. It was then reduced by a limitation on the use of streptomycin and tetracycline and the implementation of strict infection control measures, but it has still not been satisfactorily explained why other countries did not experience problems at that time to the same extend. The initial MRSA appeared to belong to a limited number of Group III phage types (53/75/77) [4] which were resistant to penicillin, streptomycin and tetracycline. This phage type was different from that of the MSSA (80/81) associated with hospital acquired infections at that time. More modern typing methods show that these initial MRSA belonged to subgroup of clonal complex 8 (CC8), sequence type (ST) 250 [5].

In 1980 the resistance to methicillin was shown to be related to a change in penicillin binding protein (PBP). This new protein, soon named PBP2, was encoded on the *mecA* gene, a gene not native to *S. aureus*. Soon an association between this gene and other resistance genes was found, leading to the discovery of the staphylococcal cassette chromosome (SCC*mec*), a mobile genetic element capable of integrating into the staphylococcal chromosome [6]. The 1980s were also the time of the emergence of MRSA strains that were both more virulent and could spread more easily than the first MRSA strains. Epidemic MRSA (EMRSA) had arrived and appeared in countries all around the world. In the beginning of the 90^{s}, more than 30 years after its first occurrence, the MRSA epidemic started to cause major health problems all over the world, the most well known clones being E-MRSA15 (CC22) and E-MRSA 16 (CC30) [7]. The "clinical" emergence of MRSA led to an increase of staphylococcal infections in hospitals. The mortality rate for bacteremia with MRSA was shown to be higher than that for MSSA (UK data mortality 40-50%, attributable mortality 20%). Patients with a MRSA bacteremia had a 1.5 fold longer length of stay and 2-fold increase in cost of hospitalisation [8]. Although there are not many robust statistics on the actual cost of MRSA, the estimated extra healthcare insurance costs in the US are estimated at 30 billion dollars per year. In the data of the European epidemiological network EARSS from 1998-2000, only a few countries were exempt from this epidemic: only in Denmark, Finland, Iceland, the Netherlands and Sweden fewer than 2% of *S. aureus* blood culture isolates were MRSA [9]. In the Netherlands this was thought to be due to the nation-wide implementation of a rigorous search and destroy policy in 1983, entailing screening and isolation of all patients from foreign hospitals and the treatment –where possible- of MRSA carriage, in combination with a restrictive antibiotic use in humans.

However, in spite of control efforts, MRSA is increasing even in the Netherlands. *Staphylococcus aureus* is once again showing its adaptable nature with another change in epidemiology. In 1982 the first community associated MRSA (CA-MRSA) outbreak was described in a community of intravenous drug users [10]. At first CA-MRSA infections were limited to closed populations such as Native Americans, prison inmates and children attending day care centers, but lately most CA-MRSA infections

occur in people without specific risk factors. The term CA-MRSA was at first no more than an epidemiological description for infections occurring in people outside of health care institutions but it became more and more clear that there were differences between health care associated MRSA (HA-MRSA) and CA-MRSA infections. These included the genetic profile of the bacteria involved and the type of infections, with the majority of CA-MRSA infections being skin and soft tissue infections [11]. HA-MRSA often carried SCC*mec* cassettes of type I, II and III which are relatively large and carry resistance to many other antibiotics. In contrast, CA-MRSA predominantly carried type IV or V SCC*mec* cassettes which are much smaller and carry only resistance to methicillin. In addition to this, CA-MRSA strains appeared to carry the Panton-Valentine Leukocidin gene (PVL) more often [11]. This gene was described as early as 1894 and thus clearly predates the SCC*mec* cassette. It was further characterized by Panton en Valentine in 1932. It encodes a pore-forming toxin that acts against leukocytes and erythrocytes and is a clinically relevant virulence factor. PVL positive *S. aureus* strains are associated with skin infections, furunculosis and necrotizing pneumonia. Since the PVL gene is not linked to the SCC*mec* cassette, it is hypothesized that CA-MRSA arose via transfer of a SCC*mec* cassettes into PVL positive MSSA. The source of the SCC*mec* cassettes could be other coagulase negative staphylococci. For instance in *S. epidermidis*, in which a type IV SCC*mec* was described in 1970 or *S. haemolyticus* [12].

By now MRSA infections are no longer the quintessential nosocomial infections they once were and MRSA carriers are found both in the community and in hospitals. In order to meaningfully distinguish hospital and community associated MRSA, the best definition should not merely be an epidemiological one but should include other characteristics such as the clonal lineage and SCC*mec* type. CA-MRSA is now moving into hospitals and causing outbreaks and health care associated infections [13]. Interestingly, one of the successful CA-MRSA PVL positive clones (ST30) is a re-emergence of the previously mentioned MSSA phage type 80/81, which caused severe hospital and community associated infections around the world in the 1960's. After picking up a SCC*mec* type IV cassette, this clone regained it's foothold as an important human pathogen. In fact, all of the pandemic, ecologically successful HA-

and CA-MRSA clones have successful MSSA precursors adapted for effective transmission. The presence of multiple SCC*mec* types within the same clonal lineage indicate that horizontal transfer of SCC*mec* elements might not be as not uncommon in *S. aureus* as was previously thought.

Methicillin resistant *Staphylococcus. aureus* in animals

Humans are not the only species harbouring *S. aureus*, although traditionally human and animal isolates of *S. aureus* have been seen as separate populations and classified into host-specific biovars. Until recently prevalence studies reported low levels of MRSA among staphylococci of animal origin but the number of reports on MRSA infections in animals has been increasing. Infections in horses, dogs and cats have been described in South Korea, Japan, the UK, the Netherlands, Canada and the US. These cases were predominantly due to transmission of 'human' clones to animals. For instance in a study by Loeffler et al. among staff and animals in a small animal hospital in the UK, more than 80% of isolates were identical or closely related to EMRSA-15, the UK epidemic MRSA strain [14]. In outbreaks in horse hospitals in Ireland and Canada CC8 (ST8, ST254, USA 500) was the predominant clone with transfer between staff and patients. Carriage in healthy dogs is still rare but MRSA can be found in 2% of those with skin conditions. In companion animals and horses the majority of MRSA infections is caused by "human" strains, suggesting spill over from the human reservoir into animals.

The first isolation of a methicillin resistant *S. aureus* from a veterinary specimen from a food animal was reported in Belgium from a bovine mastitis in 1972 [15]. This strain was found in over 20 dairy herds, probably as the result of clonal spread. Since then, MRSA has incidentally been reported from bovine mastitis in different countries such as South Korea and Hungary. Among 1913 culture samples from food animal origin gathered between 2001 and 2003, Lee at al. found 15 MRSA, of which 12 were from dairy cattle and 3 from chickens [16]. The authors warned about the potential health risk due to a possible spread of MRSA through the consumption of contaminated food products. Interestingly however, although there is a potential

route of transmission through milk, especially if unpasteurized, transmission to humans of bovine MRSA has seldom been described. In dairy cattle the main MRSA clones still appear to be species specific.

In 2005, Andrew Waller wrote an editorial in the Veterinary Journal titled "The creation of a new monster: MRSA and MRSI- Important emerging veterinary and zoonotic diseases", pointing our that there were increasing reports of MRSA transferring between animal species, such as humans and horses, dogs and other pets and that there was a risk of transfer of *mec*A genes to more common animal pathogens like *S. intermedius* [17]. The potential risk of food animals as a reservoir for human MRSA was not perceived important. Until 2005 only sporadic occurrence of MRSA in live stock animals, other than dairy cattle, were reported. The first appearance of MRSA in pig-farmers was reported by Aubrey-Damon et al. in France, but the observation got no attention, since it was one of many resistant micro-organisms they looked at and MRSA was not found in pigs and their corresponding farmer[18]. The first evidence that live-stock animals may be a new source of MRSA for humans came in the same year from the Netherlands.

Conclusions

MRSA in man has been a challenge to human health since its first occurrence in the 1960's when it slowly started to spread within the hospitals and since it's emergence in the 1990's in the community. Until recently, other sources of MRSA outside humans were not known to exist, although incidental transmission from animals to humans and vice versa was described. In this thesis we investigate the prevalence of MRSA carriage in people in contact with livestock and how this affects the the risk of MRSA carriage and infection of humans in the community and in health care settings. Furthermore we investigated the application of existing and new diagnostic methods in this emerging MRSA clone.

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Chapter 2: Methicillin-resistant *Staphylococcus aureus* in Pig Farming in the Netherlands

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(published Emerging Infectious Disease 2005;11:1965-6)

Abstract

We conducted a study among a group of 26 regional pig farmers to determine the methicillin-resistant *Staphylococcus aureus* prevalence rate and found it was >760 times greater than the rate of patients admitted to Dutch hospitals. While *spa*-type t108 is apparently a more widespread clone among pig farmers and their environment, we did find other *spa*-types.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major nosocomial pathogen, highly prevalent in many European countries and throughout the world (1). In the Netherlands, the prevalence of MRSA among clinical isolates is still <1%, among the lowest in Europe (1). This low prevalence is probably best explained by the national policy that entails strict screening and isolation of all persons who are considered at high risk for MRSA when admitted to a hospital. This high-risk population has essentially consisted of patients admitted to or treated in foreign hospitals. As a result of this policy for all healthcare institutions, the prevalence of MRSA in the Dutch community is extremely low as well. In a recent study among ≈10,000 patients admitted to 4 Dutch hospitals, 23% carried *S. aureus*, but only 0.03% of the isolates were methicillin-resistant (2).

In July 2004, we unexpectedly found MRSA in the preoperative screening cultures of a 6-month-old girl before thoracic surgery. Neither the girl nor her family (parents, 1 sister) had a history of travelling or admission to a foreign hospital. In the following months, the girl remained colonized with MRSA during consecutive decolonization attempts. Subsequently, the girl's parents were found to be positive for MRSA. The family lived on a farm and raised pigs.

To further investigate pig farming as a possible source of MRSA in Dutch patients, we screened a selection of pigs owned by the MRSA-positive farmer, and other regional pig farmers in November 2004. In January and February 2005, 2 new cases of MRSA were identified, one in a pig farmer from a different region and one in the son of a veterinarian who worked mostly with pigs. Subsequently, the strain was also isolated from the veterinarian and from a nurse in the hospital unit to which the son was admitted.

Although the aforementioned cases were unrelated in time and location, they shared some features. In all the cases, other family members were MRSA-positive, decolonization was repeatedly unsuccessful, and genotyping performed in the National Institute of Public Health and Environment (RIVM, Bilthoven, the

Netherlands) showed the strains were not typeable by pulsed-field gel electrophoresis (PFGE) with restriction endonuclease *Sma*I (the standard method).

The study

Initially, the nares of 10 pigs were cultured. All were negative for MRSA. At a later stage, the perineum of 30 pigs was cultured; 1 was positive for MRSA. The regional pig farmers were screened (throat and nares) during a monthly professional meeting that happened to be on the farm of the MRSA-positive family, at the time of investigation. With the exception of this meeting, the farmers had no further epidemiologic links, other than being from the south-eastern region of the Netherlands. Six (23%) of the 26 farmers were colonized with MRSA.

As mentioned above, all MRSA isolates were resistant to digestion with restriction-endonuclease *Sma*I, when typing with PFGE was attempted. To ensure that we did not falsely classify a pig-related staphylococcal species as MRSA, the identification of all isolates was confirmed by testing for the presence of a *S. aureus*–specific DNA element as well as the *Mec*A gene, according to the methods of Reischl et al. (3). To compare the MRSA isolates, we performed random amplified polymorphic (RAPD) DNA analysis with primers Eric II (5´-AAG TAA GTG ACT GGG GTG AGC G-3´), RW3A (5´-TCG CTC AAA ACA ACG ACA CC-3´), D14307 (5´-GGT TGG GTG AGA ATT GCA CG-3´) and *spa*-typing.

Overall, 3 different MRSA strains were identified. The isolates of the girl (case-patient A), her parents, and the pig from their farm were identical with random amplified polymorphic DNA and belonged to *spa*-type t108. Furthermore, one of the regional pig farmers screened during the meeting, the pig farmer from a different region (case-patient B), the young boy (case-patient C), as well as his father and the nurse who treated the boy, were colonized with the same strain (Table 1). Three of the regional pig farmers shared *spa*-type 567. The isolate from the remaining MRSA-positive regional farmer showed a *spa*-type not previously described (Table 1).

Table 1. Molecular typing of methicillin-resistant *Staphylococcus aureus* isolates

Case-patients	***Date of culture***	***RAPD* type***	**Spa*-type***
Patient A (girl)	Jul 2004	A	t108
Regional farmer 1 (father of patient A)	Aug 2004	A	t108
Mother of patient A	Nov 2004	A	t108
Pig	Feb 2005	A	t108
Patient B (farmer, different region)	Jan 2005	A	t108
Patient C (boy)	Feb 2005	A	t108
Father (veterinarian) of patient C	Feb 2005	A	t108
Nurse of patient C	Feb 2005	A	t108
Regional farmer 2	Nov 2004	Not done	t108
Regional farmer 3	Nov 2004	Not done	t567
Regional farmer 4	Nov 2004	Not done	t567
Regional farmer 5	Nov 2004	Not done	t567
Regional farmer 6	Nov 2004	Not done	t943

* random amplified polymorphic DNA analysis

Conclusions

Recently, MRSA has been found in horses and in persons who take care of them (4). Human carriage has also been linked to colonized companion cats and dogs (5,6) While Lee et al. (7) reported an MRSA isolation frequency of 0.6% in major food animals, but did not find MRSA in 469 samples from pigs, Armand-Lefevre et al. (8) described *S. aureus* (methicillin-susceptible and -resistant) carriage among pigs and pig farmers. Although the authors showed that both farmers and pigs carried methicillin-sensitive *S. aureus* and MRSA and that both groups shared certain multilocus sequence typing, the isolates came from separate, nonrelated collections.

Here we demonstrate transmission of MRSA between an animal and human (pig and pig farmer), between family members (pig farmers and their families), and between a nurse and patient in the hospital. The unexpected high frequency of MRSA among the group of regional pig farmers (>760× higher than in the general Dutch population) indicates that their profession might put them at risk for MRSA colonization. Overall, we found 3 different MRSA strains, including a new *spa*-type. Therefore, we expect that multiple strains are present in the pig population and the pig farmers. The strain with *spa*-type t108 appears to be more prevalent and widespread, given that the strain spread from animal to human, between family members, between patient and nurse, and among pig farmers from different regions.

Further research on a larger scale is needed to see if these observations hold true in other regions. If so, pig farming poses a significant risk factor for MRSA carriage in humans that warrants screening wherever pig farmers or their family members are admitted to a hospital.

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Chapter 3: Methicillin-resistant *Staphylococcus aureus* in Veterinary Doctors and Students, the Netherlands

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Abstract

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in the Netherlands, at 1.0%, is among the lowest in Europe. In 2004, a relationship between pig farming and a high risk for MRSA carriage was found. To investigate if those in professional contact with livestock are at higher risk for MRSA carriage, we screened 80 veterinary students and 99 veterinarians and questioned them about animal contacts and known MRSA risk factors.. We found 7 carriers of MRSA, a prevalence of 4.6%, which is similar to that found in patients who had previously been treated at foreign hospitals. A correlation of MRSA carriage with a specific animal group could not be established. To preserve the low prevalence of MRSA in the Netherlands, persons involved in the care of livestock should be isolated and screened on admission to the hospital.

Introduction

In the Netherlands, the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical isolates of *S. aureus* has been <1% during the past decade (1,2) and, at 1.0%, remains one of the lowest in Europe (3). This low prevalence is best explained by the national "search and destroy" policy, which demands isolation and screening of patients at risk for MRSA carriage on admission to healthcare facilities. So far, the patients at risk have mainly been persons who had previously been admitted to or treated in foreign hospitals.

In 2004, three patients in our hospital who had no relation to foreign hospitals or exposure to other known sources of MRSA were unexpectedly found to carry MRSA. The patients were a pig farmer, a pig farmer's child, and a veterinarian's child. A subsequent screening of local pig farmers showed MRSA prevalence of >20%, which suggested that contact with pigs, at least in that region of the Netherlands, posed a substantial risk of acquiring MRSA (4). If that hypothesis were true, isolation on admission and screening of pig farmers and their family members for MRSA would be indicated. To further investigate if those in professional contact with livestock are at higher risk for MRSA carriage, we screened a selection of veterinary doctors and students.

Materials and Methods

We screened 80 veterinary students in the last phases of their education and 99 veterinarians attending a conference on livestock. Cultures were taken from both anterior nares and throat. All participants were asked to fill in a questionnaire about the type of animal contacts and possible exposure to known MRSA risk factors.

We incubated all cultures in a salt-enriched nutrient broth and after 24 hours subcultured them on blood agar plates and MRSA-ID agars (bioMerieux, La Balme Les Grottes, France). Colony morphology and latex agglutination test (Staphaurex, Remel, Lenexa, KS, USA) initially identified staphylococci; cefoxitine-disc diffusion determined methicillin resistance, according to Clinical and Laboratory Standards

Institute criteria (5). All cefoxitine-resistant isolates underwent further identification and susceptibility testing to cefoxitine, gentamicin, vancomycin, teicoplanin, clindamycin, erythromycin, rifampicin, ciprofloxacin, cotrimoxazol, and tetracycline, using the Phoenix Automated Microbiology System (Becton Dickinson, Franklin Lakes, NJ, USA). We also performed *mec*A gene PCR, typing by pulsed-field gel electrophoresis (PFGE) with *Sma*I (the standard method), and *spa*-typing on all cefoxitine-resistant strains.

Results

The main characteristics of the veterinary doctors and students are listed in Table 1. Among the 179 persons tested, 7 (3.9%) MRSA carriers were found: 2 students and 5 veterinarians (Table 2). MRSA carriage varied depending on whether or not study participants had contact with livestock. MRSA carriage was 4.6% among 152 students and doctors in contact with livestock and 0% among 27 students who reported no contact with livestock. All MRSA carriers in this study had recent or regular contact with pigs and cows; only 3 veterinarians reported regular contact with sheep. Because all carriers reported contact with cows and pigs, no relative risk could be calculated (Table 3). In each group, 1 person indicated a known risk factor for MRSA carriage (1 had been admitted to a foreign hospital; 1 had an MRSA-positive family member), but both tested MRSA negative.

In addition to 7 MRSA isolates, *S. sciuri* was isolated from 1 veterinarian. This strain showed green colonies on the ID-MRSA plates and was Staphaurex positive, which caused the risk to be wrongly identified as MRSA.

All cefoxitine-resistant isolates were susceptible to vancomycin, teicoplanin, rifampicin, and ciprofloxacin, but all were resistant to tetracycline. All MRSA strains and the *S. sciuri* were *mec*A positive and were resistant to digestion with restriction endonuclease *Sma*I when typing by PFGE was attempted, similar to the strains described by Voss et al. (4). Overall, 3 different MRSA types were identified by spa typing; 2 students and 1 veterinarian carried *spa*-type t011, 3 veterinarians carried

spa-type t108, and 1 veterinarian carried *spa*-type t034. In contrast to the study of Voss et al., t108 was not a dominant *spa*-type.

Table 1. Main characteristics of veterinary students and veterinarians, the Netherlands

Characteristics	**Veterinary students n = 80, no. (%)**	**Veterinarians n = 99, no. (%)**
Mean age (range), y	26 (23–41)	43 (27–60)
Male	24 (30)	83 (83)
Professional contact limited to livestock	49 (63)	72 (73)
Professional contact limited to companion animals	27 (32)	0
Professional contact with livestock and companion animals	4 (5)	27 (27)
Contact with cows	48 (60)*	83 (83)†
Contact with pigs	37 (47)*	72 (72)†
Contact with sheep	Not known	36 (36)†
Contact with pets at home	52 (65)	81 (81)
Risk factors for MRSA carriage‡	1 (1.2)	1 (1)

*Regular contact in past 3 months.

†Regular part of practice and/or regular contact in the past 6 months.

‡MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 2. Characteristics and type of animal contact of MRSA carriers, the Netherlands*

Case	Sex	Profession	Pigs	Cows	Horses	Sheep	Companion animals
1	F	Student	X	X		?	
2	F	Student	X	X	X	?	
3	M	Veterinarian	X	X	X	X	X
4	M	Veterinarian	X	X	X		X
5	M	Veterinarian	X	X		X	
6	M	Veterinarian	X	X		X	
7	M	Veterinarian	X	X			

*MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 3. Estimates of relative risk for exposure to types of animals for veterinary students and veterinarians, the Netherlands

Type of animal	Relative risk	95% Confidence interval
Pigs*	9.0	0.52–154
Cows*	5.3	0.31–90
Sheep†	4.35	0.52–40
Companion animals	0.86	0.17–4.2
Horses	0.72	0.14–3.6

*The number of carriers without exposure in this group was 0; estimate of relative risk was made by adding 0.5 to all groups.

†Data on veterinarians only.

Discussion

MRSA has been found in various animals, such as horses (6) and livestock (7), including pigs (4,8). So far, only 1 study has indicated transmission from livestock to caretakers (4). The extent of this transmission and its clinical significance remain unknown, also undetermined is whether persons in professions other than farming are at increased risk of becoming MRSA carriers. The overall MRSA prevalence in veterinary students and doctors involved in farm animal health in the Netherlands was about 160× higher than that among patients at hospital admissions (4.6% vs. 0.03%) (9); this prevalence falls within the range of that found in patients from foreign hospitals (3.5%–5%) (10). At least with regard to the search and destroy policy in the Netherlands, veterinarians and veterinary students who come in contact with the healthcare system may therefore qualify as patients at high risk, warranting screening and isolation on admission to hospitals.

The high frequency of MRSA carriage among veterinary doctors and students is unexpected. While protective coveralls and boots are routinely used during veterinary contact with livestock, protective masks are not. Because *S. aureus* colonization and transmission occur mainly through contact from the hands to the anterior nares, the standard measures are probably insufficient to prevent MRSA colonization. Therefore, masks and gloves could be considered as additional protective measures.

Although low in comparison with several other countries, the quantity and intensity of antimicrobial use in livestock has increased in the Netherlands (11). Data from 1997 to 2004 show that the main antimicrobial classes used in livestock are tetracycline and trimethoprim sulfonamide combinations. All the MRSA strains in this study, and all the strains found by Voss et al., were resistant to tetracycline.

We conclude that veterinary doctors and students caring for livestock have a high risk of being colonized by MRSA. The percentage of MRSA carriage in the doctors and students surveyed is such that, to preserve the low prevalence of MRSA in the Netherlands, all persons involved in the care of livestock should be isolated and screened on admission to the hospital, according to national policy. Further

investigation is needed to determine the exact source of MRSA in livestock and the effect of risk factors such as the use of antimicrobial agents on MRSA carriage in livestock. This type of research should be conducted in other countries to find out if this phenomenon is limited to the Netherlands or is international.

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Chapter 4: Prevalence of methicillin-resistant *Staphylococcus aureus* among veterinarians: an international study

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Abstract

Pig farmers and veterinarians in contact with livestock in The Netherlands have a higher risk of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage than the general population. The objective of this study was to investigate whether this is also true for other professionals in contact with pigs in an international setting. A convenience sample of 272 participants at an international conference on pig health in Denmark was screened for MRSA carriage, using combined nose / throat swabs and were asked to complete a questionnaire concerning animal contacts, exposure to known MRSA risk-factors, and the protective measures taken when entering pig farms. In total, 34 (12.5%) participants from nine countries carried MRSA. Thirty-one of these isolates were non-typeable by pulsed-field gel electrophoresis following *Sma*I digestion of chromosomal DNA. All of the non-typeable isolates belonged to *spa*-types (t011, t034, t108, t571, t567 and t899) that correspond to multilocus sequence type 398. All of the abovementioned *spa*-types, with the exception of t899, have been isolated previously from either Dutch pigs, pig farmers and / or veterinarians. Protective measures, e.g., masks, gowns and gloves, did not protect against MRSA acquisition. Transmission of MRSA from pigs to staff tending to these animals appears to be an international problem, creating a new reservoir for community-acquired MRSA (CA-MRSA) in humans in Europe, and possibly worldwide. The rise of a new zoonotic source of MRSA could have a severe impact on the epidemiology of CA-MRSA, and may have consequences for the control of MRSA, especially in those countries that maintain a low prevalence by means of search-and-destroy policies.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important cause of nosocomial disease worldwide. Recent reports indicate that the epidemiology of MRSA is undergoing a major change following the emergence of community acquired MRSA (CA-MRSA) [1–3]. CA-MRSA can cause serious infections in otherwise healthy individuals and has, in some instances, even surpassed methicillin-susceptible *S. aureus* as a pathogen [4]. In 2004, contact with livestock, especially pigs, was identified as a risk-factor for MRSA carriage in The Netherlands [5]. Surveys of Dutch pig farmers [5] and veterinarians [6] showed a significantly higher MRSA carriage rate in these groups (26% and 4.6%, respectively) than in the general Dutch population (0.03%) [7]. A survey of slaughterhouse pigs showed that 39% of pigs were MRSA-positive [8].

Isolates from pigs, pig farmers and veterinarians were non-typeable by standard typing using pulsed-field gel electrophoresis (PFGE), following digestion of chromosomal DNA with *Sma*I, because of a novel DNA methylation enzyme present in these isolates [9]. Typing of these isolates showed that they belonged to a number of closely related *spa*-types (t011, t034, t108, t567 and t571), all of which corresponded to multilocus sequence type (ST) 398. Strains that were non-typeable by *Sma*I PFGE were first observed in The Netherlands in 2003 and are increasing in frequency (12th International Symposium on Staphylococci and Staphylococcal Infections, Maastricht, The Netherlands, 2006; abstract 0.26). A strict 'search-and-destroy' policy in The Netherlands has kept the prevalence of MRSA in hospitals at 1% [10–12]. In order to preserve the effectiveness of this policy, the national guidelines were recently changed so that all individuals in professional contact with pigs are now isolated and screened for MRSA upon admission to a hospital. It is currently unknown whether this new source of CA-MRSA is limited to The Netherlands, or whether it is an international problem. However, the latter is probable, since the meat and livestock market is international. In order to investigate whether contact with pigs might be a risk-factor for MRSA carriage in countries other

than The Netherlands, the present study screened a random selection of participants at an international conference on pig health in Denmark.

Materials and methods

A convenience sample of 272 individuals from among c. 2500 participants at a conference in Copenhagen, Denmark, concerning pig health was screened. One swab per individual was taken from both anterior nares and throat by either a qualified physician or by the participants themselves under the direct supervision of this physician. Each of the individuals sampled was asked to complete a questionnaire seeking information concerning profession and the type and intensity of contact with pigs, protective measures taken in pig farms, recent hospital admissions, and contact with known MRSA-positive family members. All swabs were incubated in a semi-selective Tryptone Soy broth containing NaCl 2.5% w/v, cefoxitin 3 mg/L and aztreonam 10 mg/L (SSI Diagnostika, Hillerød, Denmark). After 24 h, the broths were subcultured on sheep blood 5% v/v agar plates and MRSA-ID agar plates (bioMerieux, LaBalme Les Grottes, France). Staphylococci were initially identified on the basis of colony morphology and tube coagulase tests. Methicillin resistance was determined by disk-diffusion using cefoxitin disks according to CLSI recommendations [13]. Species identifications and susceptibility testing results were confirmed using the Vitek II Automated Microbiology System with ID card GP and AST card AST-P554 (bioMerieux), which includes susceptibility tests for ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, linezolid, quinupristin–dalfopristin, rifampicin, vancomycin, teicoplanin, trimethoprim–sulphamethoxazole and tetracycline. All cefoxitin-resistant isolates were also investigated by PCR for carriage of the *mec*A gene [14], and their staphylococcal cassette chromosome (SCC)*mec* type was determined using the primers described by Zhang et al. [15]; the isolates were also typed by PFGE following *Sma*I digestion of chromosomal DNA [16] and their *spa*-type was determined [17]. Data were analysed by univariate logistic regression analysis, with carriage of MRSA as a dependent variable, and contact hours with pigs, country of origin, protective measures and contact with cows as

independent variables. The best model was selected with the backward likelihood ratio method. If p was >0.05, the coefficient was discarded. All analyses were performed using SPSS v.12 (SPSS Inc., Chicago, IL, USA).

Results

Of the 272 participants who were screened, 34 (12.5%) carried a *mec*A-positive *S. aureus* strain. Table 1 summarises the main characteristics of the participants, together with data concerning animal contact and the use of protective clothing.

Table 1: Main characteristics of the 272 participants screened for carriage of MRSA.

	Non-carriers (238)	**MRSA carriers (34)**	**p-value****
Mean age (yrs)	42 (range 22-69)	42 (range 28-57)	NS
Male	166 (70)	27 (80)	NS
Female	65 (28)	4 (12)	NS
Unknown*	7 (3)	3 (9)	NS
Type of profession			
Veterinarian	202 (84)	33 (97)	NS
Commercial	8 (4)	0 (0)	NS
University	9 (4)	1 (3)	NS
Research	7 (3)	0 (0)	NS
Student	5 (2)	0 (0)	NS
Other	7 (3)	0 (0)	NS
Frequency of pig contact#			
Frequent	113 (47)	32 (94)	0.0001
Sometimes	83 (35)	2 (6)	0.0003
Seldom	42 (18)	0	0.0001

	Non-carriers (238)	**MRSA carriers (34)**	**p-value****
Use of protective equipment			
Gown	145 (61)	17 (50)	NS
Gloves	113 (47)	25 (74)	0.02
Mask	74 (31)	19 (56)	NS
Type of animal contact			
Pigs	238 (100)	34 (100)	NS
Dairy cows	61 (26)	18 (53)	0.002
Meat cows	36 (15)	7 (20)	NS
Poultry	24 (10)	1 (3)	NS
Sheep	29 (12)	5 (15)	NS
Goats	11 (5)	0 (0)	NS
	Non-carriers (238)	*MRSA carriers (34)*	*p-value***
Horses	39 (16)	4 (12)	NS
Companion animals	96 (40)	10 (30)	NS
Pets at home	157 (66)	21 (62)	NS
Recent hospital stay	5 (2)	1 (3)	NS
MRSA-positive family member	5 (2)	1 (3)	NS

Numbers in parenthesis are percentages**.**

*Not filled in on survey form

**p-values were determined by Fishers exact test

#Frequency of pig contact:

frequent =daily and/or more than 5 hours per week

sometimes = less than 5 hours/week but with a minimum of once per month

seldom = less than once/month

Table 2 shows the number of participants and MRSA carriers according to country. Thirty-one of the 34 isolates were non-typeable by PFGE following *Sma*I digestion of chromosomal DNA. However, spa typing of these 31 isolates revealed that 26 belonged to closely related *spa*-types (i.e., t011, t034, t108, t571, t567), all of which were shown in previous studies to correspond to ST398 [8] (Table 2). The remaining isolates belonged to *spa*-type 899, which was also shown by multilocus sequence typing (MLST) to belong to ST398. Isolates of *spa*-type t011 were isolated from participants from four different European countries; *spa*-type t034 was isolated from the two delegates from outside Europe. The three isolates that were typeable by PFGE belonged to *spa*-types t022, t111 and t1730, and were recovered from Danish, French and Italian delegates, respectively; *spa*-type t022 corresponds to ST22, and *spa*-type t111 corresponds to ST5. All 34 isolates were susceptible to vancomycin, rifampicin, quinupristin–dalfopristin, fusidicacid and linezolid. Further resistance phenotypes of the 31 ST398 MRSA isolates are shown in Table 3. Nine (29%) isolates were resistant to four antibiotic classes, and 18 (58%) to five or more antibiotic classes. All isolates were tetracycline-resistant, 22 (70%) isolates had an MLSB phenotype, and 15 (48%) were resistant to trimethoprim–sulphamethoxazole. The four isolates that were resistant to ciprofloxacin were from Italy (2) and Spain (2). There was no clear association between the *spa*-type and the resistance pattern. The most frequent SCC*mec* type was SCC*mec* V (n = 24, 70.6%), followed by type IVa (n = 3) and type III (n = 2). No SCC*mec* type could be assigned for five isolates when the primers described by Zhang et al. [15] were used.

Univariate analysis, with MRSA carriage as the endpoint, showed a significantly increased risk of MRSA carriage for individuals having frequent (daily or a minimum of 5 h / week) pig contact, as compared with those seldom having contact (less than once per month), with an OR of 16.3 (CI 3.75–70.6). Individuals with infrequent contact (<5 h / week, but a minimum of once per month) had a non-significant trend towards a higher risk, with an OR of 2.4 (CI 0.58–9.8, p not significant) as compared with those seldom having contact. Contact with cows, country of origin, and use of protective measures, especially wearing of a mask, had no influence on

the rate of MRSA colonisation. Indeed, statistically, not wearing a mask was protective, with an OR of 0.38 (CI 0.12–0.99).

Table 2. Numbers of participants per country and the distribution of MRSA carriers among countries including *spa*-types.

Countries	Participants / country	MRSA carriers / country (%)	*spa*-types (n)[#]
Australia	6	0	-
Austria	8	0	-
Belgium	6	1 (16)	t011(1)
Brazil	8	0	-
Bulgaria	1	0	-
Canada	16	1 (6)	t034 (1)
Cyprus	1	0	-
Czech	5	0	-
Denmark	29	1 (3)	t022 (1)
Finland	4	0	-
France	6	1 (16)	t111(1)
Germany	39	13 (33)	t011 (8), t034 (4) t108 (1)
Ireland	5	0	-
- Continued -			
Italy	13	8 (61)	t108 (1), t899(5) t1730 (1)
Japan	1	0	-
Korea	1	0	-
Lithuania	2	0	-
Malaysia	1	0	-
Malta	1	0	-
Mexico	3	0	-
Netherlands	26	6 (23)	t011(3);t108(1);t56

Countries	**Participants / country**	**MRSA carriers / country (%)**	***spa*-types (n)[#]**
			7(1); t571(1)
New Zealand	2	0	-
Norway	5	0	-
Philippines	1	0	-
Poland	3	0	-
Portugal	3	0	-
Serbia	2	0	-
Slovakia	1	0	-
South Africa	3	0	-
South Korea	1	0	-
Spain	11	2 (18)	t011 (2)
Sweden	12	0	-
Switzerland	12	0	-
Taiwan	1	0	-
Thailand	9	1 (11)	t034 (1)
United Kingdom	8	0	-
USA	14	0	-
Vietnam	2	0	-

number of isolates with given *spa*-type

Table 3 Resistance phenotypes of the 31 MRSA isolates that were non-typable by PFGE with *Sma*I and that belong to ST398.

Isolates are depicted from least resistant to most resistant. Nine isolates (29%) are resistant to four antibiotic classes, 18 (58%) are resistant to five or more antibiotic classes. All isolates are tetracycline resistant, 22 isolates (70%) have an MLS_B-phenotype, 15 (48%) are resistant to trimetoprim/sulphamethoxazole. The four isolates that are resistant to ciprofloxacin are from Italy (2) and Spain (2). There is no clear association between *spa*-type and resistance pattern.

*Isolate**	*country of residence*	*spa-type*	*Cipro-floxacin*	*Clinda-mycin*	*Erythro-mycin*	*Genta-micin*	*Tetra-cycline*	*SXT $^{\$}$*
13	Germany	t011	S	S	S	S	R	S
28	Netherlands	t567	S	S	S	S	R	S
29	Netherlands	t108	S	S	S	S	R	S
1	Thailand	t034	S	S	S	S	R	R
6	Canada	t034	S	S	S	R	R	S
14	Italy	t899	I	R	S	S	R	S
27	Netherlands	t571	S	S	S	S	R	R
2	Italy	t899	S	R	R	S	R	S
4	Spain	t011	R	R	S	S	R	S
5	Germany	t011	S	R	R	S	R	S
8	Belgium	t011	S	S	S	R	R	R
9	Italy	t108	S	R	R	I	R	S
15	Germany	t011	S	R	R	S	R	I
18	Germany	t011	S	R	R	S	R	S
26	Germany	t011	S	R	R	S	R	S
30	Germany	t011	S	R	R	S	R	S
3	Spain	t011	R	R	R	S	R	S
7	Germany	t034	S	R	R	S	R	R
10	Germany	t034	S	R	R	S	R	R
11	Germany	t034	S	R	R	S	R	R
12	Germany	t108	S	R	R	S	R	R
17	Italy	t899	S	R	R	S	R	R
19	Germany	t011	S	R	R	R	R	S
20	Italy	t899	S	R	R	S	R	R
23	Germany	t011	S	R	R	S	R	R
24	Germany	t034	S	R	R	S	R	R

*Isolate**	*country of residence*	*spa-type*	*Cipro-floxacin*	*Clinda-mycin*	*Erythro-mycin*	*Genta-micin*	*Tetra-cycline*	*SXT$*
25	Netherlands	t011	S	R	R	S	R	R
31	Italy	t899	R	R	R	S	R	S
16	Netherlands	t011	S	R	R	R	R	R
21	Italy	t899	R	R	R	S	R	R
22	Netherlands	t011	S	R	R	R	R	R

*Isolate numbers are not identical to participant numbers.
$ SXT = trimethoprim/ sulphamethoxazole

Discussion

Community-acquired MRSA is rapidly becoming a widespread pathogen worldwide, primarily as a cause of skin and soft-tissue disease, but sometimes of invasive infection, e.g., necrotising pneumonia, in otherwise healthy individuals [1–4]. The source of CA-MRSA is unknown, but clinical and molecular epidemiological studies have indicated two separate evolutionary pathways for CA-MRSA and hospital-acquired MRSA. MRSA strains belonging to several different multilocus sequence types have been associated with infection and colonisation in both humans and animals, suggesting bidirectional transmission [18–26]. However, most reports are anecdotal or describe outbreaks in a single institution or country. In general, the rate of colonisation with MRSA among non-hospitalised individuals is very low [27]. In The Netherlands, the prevalence of MRSA upon admission to a hospital was 0.03% [7]. Even in countries with a high prevalence of MRSA, e.g., the USA and Portugal, carriage rates in the general population are only 0.2–3% [27–30]. For this reason, the high prevalence of MRSA carriage (12.5%) among attendees at an international conference on pig health is of great concern and, combined with the significant association between the time spent on pig farms and the risk of colonisation, indicates that contact with pigs could be an important source of MRSA carriage. Of the 34 MRSA carriers in the present study, 31 veterinarians from seven countries carried a strain that was non-typeable by PFGE. The nontypeable isolates belonged to *spa*-types (t011, t034, t108, t571, t567, t899) that correspond to ST398. All of the

above-mentioned *spa*-types, with the exception of t899, have also been found either in Dutch pigs, pig farmers and/or veterinarians. Carriage of a methicillin-susceptible ST398 strain by pigs and pig farmers has been described previously [31], suggesting that this clone is capable of colonising both pigs and humans. The source of MRSA in pigs is presently unknown, but dissemination of MRSA among pigs could be facilitated by the trade of live animals among different countries and by the use of antibiotics for mass treatment of livestock. All of the isolates in the present study were resistant to tetracycline, which is one of the main antibiotics used in pig farming in The Netherlands (http://www.cidc-lelystad.wur.nl/NL/publicaties/rapporten/maran/).

Of further concern is the fact that 58% of the ST398 isolates were truly multi-resistant, in the sense that they were resistant to five or more classes of antibiotic (Table 3). Selection of multidrug-resistant microorganisms of clinical relevance in humans has been associated previously with antibiotic consumption by livestock. A reservoir of vancomycinresistant enterococci was discovered among pigs and poultry, and led to a ban on the use of the glycopeptide avoparcin as a growth promoter in animals [32]. Later, a high proportion of poultry farmers were found to be carrying vancomycinresistant enterococci [33].

A more severe challenge is presented by MRSA, since it is a much more virulent microorganism than vancomycin resistant enterococci. The protective measures taken by veterinarians did not prevent them from becoming colonised with MRSA. This could be a result of breaches in adherence to these measures, e.g., poor hand hygiene after removal of gloves or the reuse of contaminated dust masks, or because of contamination outside pig farms. Gibbs et al. [34] showed that antibiotic-resistant bacteria from the environment of pigs, including ampicillin- and tetracycline-resistant *S. aureus*, could be recovered up to 150 m downwind of an (open) pig-breeding facility. The possibility that airborne MRSA can colonise veterinarians or other individuals in the direct vicinity of a pig farm can therefore not be excluded. When the allelic profile of ST398 is compared with predominant clones in Europe by means of the MLST database, there is no relationship with epidemic

healthcare-associated MRSA or common CA-MRSA at the present time. The situation in The Netherlands shows an increasing prevalence of ST398 among MRSA isolates from all sources. This clone has also been reported in Germany from cases of ventilator-associated pneumonia [35] and in infections in Denmark (R. Skov, unpublished data). In the present study, participants from The Netherlands, Germany, Spain, Belgium, Canada, Thailand and Italy carried 'pig-related' MRSA strains, thereby indicating that these strains are far more widespread than reported previously. If these strains are allowed to spread freely among pigs, and from pigs to humans, they could constitute an important new source of CA-MRSA. Apart from the fact that individuals in contact with pigs have a higher risk of developing MRSA infection, the high rate of carriage also has an economic effect on search and-destroy policies for MRSA because of the extra screening and isolation measures required. The high carriage rate of 'pig-related' MRSA among professionals in contact with pigs indicates that livestock may serve as an important source of CA-MRSA in Europe, and possibly worldwide. The rise of a new 'zoonotic' source of MRSA could have a severe impact on the epidemiology and control of CA-MRSA, especially in countries currently using a search-and-destroy policy. In order to preserve the low prevalence of MRSA in such countries, and to prevent a further increase of CA-MRSA in others, it is important to know the extent to which these strains may have spread in livestock and in the community, and whether screening for MRSA in individuals in contact with pigs is necessary and cost-effective.

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Chapter 5: Carriage of Methicillin Resistant *Staphylococcus aureus* in organic versus regular pig farmers

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Abstract

Methicillin resistant *Staphylococcus aureus* has been found in pigs and in people in contact with pigs. In this study organic farmers, who use less antibiotics, had a significantly smaller chance of being colonized with MRSA than regular pig farmers.

Introduction

Until recently methicillin-resistant *Staphylococcus aureus* (MRSA) was considered an important pathogen mainly in the hospital setting. Lately, this has changed and outbreaks of the so-called community-associated MRSA (CA-MRSA) have been reported all over the world [5]. Since 2003 a new clone of MRSA has been found in farm animals (pigs and veal calves) and humans in direct or indirect contact with these animals, in the Netherlands [6,10,11,12]. This clone distinguishes itself from other MRSA strains in the Dutch national collection in that it cannot by typed by pulsed field gel electrophoresis (PFGE) using the standard restriction endonuclease (*Sma*I) [1]. *Spa*-typing and multi-locus sequence typing (MLST) showed that isolates of this MRSA clone, frequently referred to as non-typable (NT)-MRSA, belong to a number of closely related *spa*-types, all of which correspond to MLST ST398. In the initial observation of this problem, Voss et al. found 26% of a selective group of pig farmers to carry NT-MRSA [11]. In a study by de Neeling et al, 39% of all slaughterhouse pigs were found to be MRSA positive [8]. Moreover on a herd level, 44 (81%) herds out 54 harboured positive pigs. It is of note that all strains found so far, are tetracycline resistant, probably due to the selective pressure of oxytretracylines, which are the most frequently used antibiotics in pig farming. If the frequent use of antibiotics contributes to the spread of MRSA among pigs, farms using less antibiotics should theoretically have lower rates of MRSA.

In the Netherlands, organically raised pigs are allowed only a single course of antibiotics in their life. Organic farmers therefore use similar antibiotics but in lower amounts than there colleagues in regular pig farming. In December 2006, we compared two groups of pig farmers, one organic and the other regular, to evaluate if the rate of MRSA carriage differed between the two groups.

Material and Methods

All farmers screened were participants of regional study-groups, that meet on regular basis to discuss farming practices. A total of 26 regular and 27 organic farmers were tested, after verbal consent. One swab per person was taken from

both anterior nares and throat by either an infection control nurse or a physician. All swabs were inoculated in a salt-enriched nutrient broth and sub-cultured on blood agar plates and MRSA-ID agars (bioMerieux, La Balme Les Grottes, France) after 24 hours of enrichment. Staphylococci were initially identified by colony morphology and latex agglutination test (Staphaurex, Remel, Lenexa, USA). Methicillin-resistance was determined by cefoxitine-disc-diffusion according to CLSI criteria and confirmed by *mec*A gene PCR [2,9].

Results

Thirteen (50%) of 26 regular farmers were MRSA positive, whereas only three of the 27 organic farmers (11%) carried MRSA. Regular pig farmers had a significantly higher chance of being colonized with MRSA (OR=8, $p<0.01$). Twenty-five of the 27 organic farmers included in this surveillance study had a closed farm where no gilts are purchased. The organic farmers had an average of 110 sows which makes them relatively large for organic farmers but still smaller than regular farmers who have on average 260 sows in the Netherlands.

Discussion

Organic farmers have a significantly lower prevalence of colonization with MRSA than regular pig farmers. Although we did not test animals on the individual farms of the organic farmers, this result could reflect a lower prevalence of MRSA in organic pigs. While these are the first data with regard to MRSA, it is well known, that resistance levels in other pathogens (*Campylobacter coli* and *Escherichia coli*) from organically raised pigs are lower than those from conventionally raised slaughter pigs [7]. The fact that 11% of organic farmers were positive, suggests that at least part of the organic pigs are positive. This might be due to other farm visitors (veterinarians, feed supplier, artificial insemination practitioners) in contact with organic and regular pig farms, through buying replacement stock or by contamination of the farm environment/animals at a time before switching to organic farming (e.g. one of the

positive organic farmers had been keeping pigs for twenty years but only switched to organic farming four years ago) [3].

Since carriers of NT-MRSA in general get colonized through contact with positive animals, the results presented are a first indication that the prevalence of NT-MRSA in organically raised pigs is lower, most probably due to reduced antibiotic consumption in organic farming, which in our setting was the most obvious difference in farming practice between the two groups [4,6,11,12]. Further field studies are warranted to confirm this hypothesis.

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Chapter 6: MRSA carriage in healthcare personnel in contact with farm animals

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Abstract

In the Netherlands it has been shown that people in contact with pigs have a higher risk of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage than the general population. Isolates of closely related *spa*-types, corresponding to multilocus sequence type (MLST) ST398, were found in pig farmers, pig veterinarians and pigs. The objective of this study was to investigate whether contact with pigs and veal calves or other livestock is a risk factor for MRSA carriage in Dutch healthcare workers (HCWs). HCWs at four general hospitals and one university hospital were asked to fill in questionnaires covering contact with animals and to take MRSA cultures of their throat and nares. Cultures of HCWs in contact with livestock were processed with samples from HCWs with no contact with livestock as controls. Seventy-seven of 1721 HCWs (4.4%) reported direct or indirect contact with pigs and/or veal calves and 145 reported contact with other livestock animals. The MRSA carriage rate in the group in contact with pigs and veal calves was 1.7% and in the control group was 0.15%. No carriers were found among HCWs in contact with other livestock. An estimated 3% of hospital staff working in Dutch hospitals serving rural populations belong to a high risk group for MRSA carriage according to the Dutch guidelines. Although MRSA carriage in HCWs in contact with livestock is 10-fold higher than in other HCWs, the difference is not statistically significant.

Introduction

In the Netherlands, the rate of methicillin-resistant *Staphylococcus aureus* (MRSA) infection in hospitals is still low.[1] This is thought to be due to restrictive use of antibiotics in combination with a strict control policy, called 'search and destroy', as described by the Dutch Working Party for Infection Prevention (WIP; www.wip.nl). The key elements of this policy are active searching and isolation of MRSA-positive patients, combined with screening of healthcare workers (HCWs) who are at risk of MRSA carriage. HCWs who carry MRSA are not allowed to work with patients until they have been cleared. Until now, this policy has been both successful and cost-effective in controlling the spread of MRSA in The Netherlands and maintaining a low prevalence.[2] Recently, Dutch investigators have shown that direct contact with pigs and veal calves is associated with an elevated risk of MRSA carriage.[3-6] All of these strains were non-typable using pulsed-field gel electrophoresis (PFGE) with *Sma*I (NT-MRSA) and belonged to MLST type ST398.[3-7] Transmission between persons, for example, between family members, has been demonstrated.[3-6] In July 2006, these findings led to an amendment of the national MRSA guideline (published on www.wip.nl). Patients who come into contact with live pigs or veal calves are now isolated and screened on admission to a hospital. It is known that HCWs are involved in transmission of MRSA between patients and there have been several reports strongly implicating them as a source of nosocomial MRSA infections.[8-14] The new guideline immediately raised the question of managing HCWs who come into contact with livestock or who have family members working with pigs and/or veal calves. In order to gain insight into the proportion of HCWs in contact with livestock and to determine the rate of MRSA carriage in this group, we studied HCWs in five Dutch hospitals.

Methods

A prospective surveillance study was performed in which random samples were taken among hospital staff in one university and four general hospitals in the south of The Netherlands. This area has a relatively high density of pig farms. HCWs

involved in direct patient care were asked to complete a questionnaire and to take a swab of their throat and nares. In hospitals 1, 3 and 4, HCWs were approached directly by infection control practitioners; in hospitals 2 and 5 approach was by mail. Based on the questionnaires they were categorised with regard to animal contact and grouped into three categories. HCWs in contact with veal calves and pigs, or who had family members working with pigs or veal calves, were placed in category 1; HCWs in direct or indirect contact (through family members or living environment) with other livestock animals in category 2; and those who reported no contact with livestock in category 3. All swabs of participants who belonged to categories 1 and 2 were processed. In each hospital, for each HCW who belonged to category 1, screening cultures from a minimum of two HCWs of category 3 (selected randomly from cultures submitted on the same day as those submitted by HCWs in category 1 or 2) were processed. Cultures were done in accordance with the standard methods used in the hospital, in accordance with the national guideline for the laboratory detection of MRSA.

Isolates resistant to cefoxitin were confirmed by *mec*A gene polymerase chain reaction.[15,16] MRSA isolates were *spa*-typed.[17] Binominal 95% confidence interval (CI) around proportions was calculated using Fleiss approximation.[18] Categories were compared with each other by using $\chi 2$-test, Fisher's exact test or t-test as appropriate.

Results

In total, 2367 survey forms were distributed and 1721 forms including screening swabs were returned (overall response rate: 73%). One survey was lost, 11 were rejected because essential questions about animal contact were not completed or the respondent did not work in patient care. The response rate was different for the five hospitals and partly depended on the method of recruitment (Table 1). Seventy-seven HCWs (4.4%) reported contact with either pigs or veal calves, of whom 57 had contact themselves and 20 had family members who worked with these animals. The main characteristics of HCWs belonging to category 1, compared with those of

category 2 (contact with livestock other than veal calves and pigs) and 3 (no livestock contact) are shown in Table II. Overall, two MRSA-positive HCWs were found; one from category 1 (contact with veal calves) and one from category 3 (control group). The MRSA carriage rate in category 1 was 1.3% (95% CI:0.07-8.0) and in the control group (category 3) was 0.16% (0.01-1.0). No carriers were found among HCWs of category 2 (0-3.2%).The isolate carried by the HCW in contact with veal calves was non-typable by PFGE with *Sma*I, *spa*-type t108. Unfortunately the isolate carried by the HCW in category 3 did not survive storage by freezing.

Table 1. Proportion of healthcare workers in contact with livestock at each hospital

Hospital	Total no. of surveys distributed	No. of completed surveys returned	No. of cultures processed	No. of HCWs in Category 1[#](%)	No. of HCWs in Category 2[§] (%)
1	693	693	139	20 (2.9)	47 (6.8)
2	500	336	114	13 (3.9)	33 (9.8)
3	186	173	173	15 (8.6)	20 (11.6)
4	288	281	261	8 (2.8)	32 (11.4)
5*	700	238	166	21 (8.8)	13 (5.5)
Total	2367	1721	855	77 (4.4)	145 (8.4)

*An excess of surveys were distributed to each ward, so no response rate could be determined.

[#] Category 1: Health care workers (HCWs) in contact with veal calves and pigs, or with family members working with pigs or veal calves.

[§] Category 2: HCWs in direct or indirect contact with other livestock animals.

Discussion

From the results of our survey, we estimate that 3% (95% CI:2.6-4.1) of hospital staff working in Dutch hospitals serving a rural population in the south of the Netherlands are in contact with pigs or veal calves. If they were patients, they would be deemed at risk of carrying MRSA, as defined by the Dutch Working Party for Infection Prevention.[2] This estimate is based on the three hospitals with a known response rate (nos. 1, 3 and 4). Hospitals 3 and 5 had the highest rate of HCWs in contact with pigs and/or veal calves which can either be explained by the high number of pig farms in the area or the fact that HCWs who felt themselves 'at risk' were more motivated to participate, or both.

The MRSA carriage rate found in HCWs in contact with veal calves and pigs was not statisticallysignificantly higher than in the control group and the carriage rate in this control group was similar to that found in the general Dutch population (0.03-0.08%).[19,20] The rate was statistically significantly lower than in patients who reported working with veal calves and/or pigs, or living at a pig or cattle farm, and who are therefore screened on admission to two of the participating hospitals (numbers 1 and 4) (25.2%, 95% CI: 17.8-34.3; unpublished data)[21], but it was not statistically significantly lower than that found in Dutch veterinarians reporting contact with pigs or veal calves (4.6%, 2.0-9.6).[4] This might be explained by failure to ask about the nature and extent of contact with the animals; in a study of veterinarians, there was a relationship between time spent in stables and risk of colonisation.[5]

We conclude that MRSA carriage of animal-related strains among HCWs in contact with pigs and veal calves does not appear to be common but carriage and subsequent risk of transmission cannot be excluded. Further investigation is needed among those HCWs with a high level of exposure to pigs and veal calves to determine whether they have a higher risk of MRSA carriage. Research on this topic needs to be conducted thoroughly as the impact on the HCWs involved can be large, resulting in exclusion from working with patients, but this has to be weighed against the risk of patients becoming colonised or infected with MRSA.

Table II Main characteristics of the participants divided in the three categories.

Characteristic	Category 1 N (%)	Category 2 N (%)	Category 3 N (%)	Total	p**
Gender					NS
Male	10 (13)	18 (12)	99 (16)	127	
Female	67 (87)	127 (88)	532 (85)	726	
Age *	35.8 (11.0)	37.8 (10.4)	36.8 (11.0)	36.9	NS
Profession					*0.09†*
Nurse	60 (78)	102 (70)	499 (79)	661	
Physician	2 (3)	11 (8)	41 (7)	54	
Other HCW	14 (18)	32 (22)	86 (14)	132	
No profession indicated	1 (1)	0 (0)	5 (1)	6	
Direct contact w. livestock	57 (71)	122 (84)	0 (0)	162	*0.03*
Family member in contact w. livestock	22 (29)	23 (16)	0 (0)	43	
Other risk factors					
Skin disease	12 (16)	30 (21)	158 (25)	200	NS
Family member with MRSA	1 (1)	0 (0)	1 (0)	2	NS†
MRSA in ward	20 (26)	28 (20)	129 (21)	177	NS
MRSA carriage	1 (1)	0 (0)	1 (0)	2	NS†
Total	77	145	631	853	

* Mean, standard deviation between brackets;
** Calculated with Chi-square test
† Calculated with Fisher's exact test

Category 1, healthcare workers (HCWs) in contact with veal calves and pigs or with family members working with pigs or veal calves

§ Category 2, HCWs in direct or indirect contact with other livestock animals

¥ Category 3, control group, no livestock contact

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Chapter 7: First outbreak of methicillin-resistant *Staphylococcus aureus* ST398 in a Dutch hospital, June 2007

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Abstract

We describe the first outbreak of non-typable methicillin-resistant *Staphylococcus aureus* (NT-MRSA) on a surgical ward in the Netherlands in June 2007. Nine cases of infection and/or colonisation among patients and healthcare workers were found.

Background

In the Netherlands, the proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) among clinical isolates of *S. aureus* is still low [1], but community-acquired MRSA occurs more frequently [2]. This increase is mainly caused by so called 'non-typable' MRSA (NT-MRSA, i.e. not typable by pulsed-field gel electrophoresis (PFGE) with *Sma*1 restriction digest[3]) belonging to multilocus sequence typing (MLST) type ST398 [4].

These strains are widely disseminated among pigs, veal calves and people in contact with pigs [5-8]. An association between the use of antibiotics in pig farming and the dissemination of these strains has been suggested [6,8], since the majority of ST398 MRSA are tetracycline-resistant and oxytetracyclins are the most frequently used antibiotics in pig farming.

Transmission within families, as well as single cases of colonised healthcare workers, have been described [5]. One report indicates possible healthcare-acquired infections with a Panton-Valentine leukocidin (PVL)-positive ST398 strain in China [9], but no nosocomial transmission to multiple patients or healthcare workers has occurred in the Netherlands to date.

Outbreak description

In June 2007 MRSA was cultured from a diabetic foot ulcer of a patient on a surgical ward. Subsequent screening of contacts among patients and healthcare workers

revealed four additional patients with MRSA infection and/or colonisation and five healthcare workers who carried MRSA.

Two of the five affected patients (one with prostate carcinoma and one with a diabetic foot) were successfully decolonised with mupirocin nasal ointment, chlorhexidine wash, and treatment with trimetoprim/rifampicin,

A further colonised patient with a gastro-intestinal malignancy, and two patients with infected diabetic foot ulcers remained colonised despite several decolonisation regimens.

Of 238 healthcare workers that were screened, five were were colonised in the nose and/or throat and had no skin conditions. All five have been treated with mupirocin nasal ointment and chlorhexidine wash and successfully decolonised.

All strains were resistant to tetracycline and non-typable by PFGE. *Spa*-typing showed that all strains were *spa*-type t567. This *spa*-type corresponds to MLST type 398, a type previously found in pigs.

None of the patients had contact with pigs or veal calves. One healthcare worker lived on the grounds of a pig farm but neither she nor her partner came into contact with pigs themselves. While we presume that this health care worker was the source of the infection, this could not be proven. No permission was given to sample the pigs on this farm.

Conclusions

The NT-MRSA strain responsible for this outbreak was *spa*-type t567, which corresponds to MLST type ST398, the clonal complex to which most of NT-MRSA strains belong. This outbreak shows that transmission on a larger scale than a one on one transmission between caretaker and patient can occur with NT-MRSA in a hospital setting.

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Chapter 8: Infection and colonization with Methicillin resistant *Staphylococcus aureus* ST398 versus other MRSA in an area with a high density of pig farms.

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Abstract

To evaluate the impact of the emergence of animal related Methicillin resistant *Staphylococcus aureus* ST398 in an area with a high density of pigfarms, a retrospective analysis was performed of all MRSA isolates in the laboratory database from 2002 till 2008 including typing results and clinical data from infection control archives and patients charts. The implementation of the screening of people in contact with pigs and veal calves for MRSA led to an increase in the average number of newly identified carriers from 16/year between July 2002 and July 2006 to 148 between July 2006 and December 2008. This is a 925% increase of which 82% (108/132) was due to ST398. The majority (74%) came from targeted screening but 7% was due to unexpected findings. A wide range of infections with ST398 occurred in patients with and without contact with livestock varying from post-operative wound infections to sepsis and post-trauma osteomyelitis with an overrepresentation of *spa*-type t567 among the clinical isolates. ST398 isolates were more often multi-resistant than isolates of other *spa*-types. The emergence of MRSA ST398 led to an increase in both MRSA carriers and MRSA infections.

Introduction

The PAMM laboratory of medical microbiology has an adherence area of 800,000 people in the South-East of the Netherlands which is also an area with a high density of pig farms. The laboratory serves four hospitals and all nursing homes and general practitioners in the area. In the region, MRSA screening is performed according to the national Dutch guidelines as issued by the Working-group on Infection Prevention (WIP, www.wip.nl). Since the carriage of MRSA is the general population is low [1,2] , this policy consists mainly of screening people from foreign hospitals and/or contacts of known MRSA positive patients. In July 2006 these guidelines were changed when it became clear that contact with pigs and/or veal calves was a risk factor for MRSA carriage [3-6]. Consequently, persons in contact with these types of livestock were included in the risk groups. As of January 2007, the changed guidelines were fully implemented in the entire region. Livestock related MRSA at that point could be distinguished from other MRSA strains since they were "non-typable" by the method used by the national reference laboratory (PFGE using *Sma*1 restriction endonuclease) [7], and by the fact that it consists of a number of closely related *spa*-types which al correspond to MLST ST398 [6]. The main *spa*-types in the Netherlands are t108, t011, t567 and t034 [*8*] (https://mrsa.rivm.nl/).

To analyze the impact of both the change in guidelines and the clinical impact of livestock related MRSA in this region, a retrospective analysis of all MRSA positive individuals was performed with regard to infections and colonization of patients and health care workers.

Materials and methods

An overview of all MRSA positive cultures was made from the laboratory system between July 2002 and December 2008. Data consisted of gender, age, whether it was a targeted screening culture or a clinical isolate, patient or health care worker, typing data from the national reference laboratory and presence of PVL.

For screening cultures, only first isolates per person were included, unless a person was negative for more than one year in at least 3 sets of screening cultures and was later found to be positive with another type of MRSA. Data were collected from infection control archives, the medical microbiology consultation system and culture request forms. Established reasons for screening were transfer from either a foreign hospital or from a Dutch health care institution with an ongoing MRSA outbreak, contact with a MRSA positive patient/healthcare worker (referred to as "outbreak screening"), contact with an MRSA positive family member and –from July 2006- contact with livestock (e.g. pigs and veal calves).

If MRSA came from a clinical culture of a patient not belonging to any designated risk group, the term "unexpected MRSA" is used. For the analysis of the infection burden of other MRSA versus MRSA ST398, a further analysis was made of all clinical cultures with MRSA between January 1st 2007 and December 31st 2008, divided into urine cultures, blood cultures, sputum cultures and for swabs in skin and soft tissue infections, swabs of pre-existing wounds [trauma, diabetic foot ulcers etc] and cultures of post-operative wounds. If cultures of more than one body site were positive, the most clinically relevant sample was taken into account, e.g. if blood and wound swabs were positive, the blood culture was counted. *Spa*-type, potential risk group, presence of PVL and antimicrobial susceptibility testing including clindamycin, erythromycin, fusidic acid, gentamycin, mupirocin, rifampin, tetracycline, vancomycin, ciprofloxacin, linezolid and trimetoprim-sulfamethoxazol were collected for these isolates.

Results

MRSA positive individuals 2002-2008

A total of 640 isolates from 637 persons were identified of which 25 were excluded because of missing MRSA-typing data. The main characteristics of the 612 remaining MRSA positive individuals are shown in table 1. There were 2 patients with two different types of MRSA and one Health Care Worker (HCW) with 3 different types of MRSA.

Table 1. Main characteristics MRSA positive individuals including reason for screening.

	Other MRSA *[no.=323]*	MRSA ST398 *[no.=292]*
Male	135 (42%)	196 (67%)
Age [median]	52 (range 0-102)	43 (range 1-95)
Patients	235 (72%)	276 (94%)
Health care workers	88 (27%)	16 (6%)
Risk group:		
Foreign hospital	41	6
Livestock contact	9	224
MRSA in family	15	8*
Other Dutch HCI	6	1
Outbreak screening	166	21[$]
Reason unknown	8	3
Unexpected	78	28

* although the indication for screening was an MRSA positive family member, 4 people of one family possibly had direct contact with livestock

[$] 9 had another PFGE / *spa*-type as the index strain of the outbreak and should be considered co-incidental findings.

In order to assess the impact of ST398 on the number of MRSA positive individuals in our population without hospital transmission, an analysis was made excluding outbreak screening isolates (figure 1). As shown in this figure, the average number of newly identified MRSA positive individuals increased from 16/year between July 2002- July 2006 to 148/year between July 2006-Dec 2008, a 925% increase of which 82% (108/132) was due to ST398. From the newly identified MRSA positive patients, the majority came from targeted screening, but the "unexpected cases" e.g. patients with an MRSA from a clinical culture, mostly representing MRSA infections, also increased from an average of 9/year between July 2002- July 2006 to 28/year

between July 2006-Dec 2008. Of the 37 unexpected MRSA before July 2006, 6 (16%) were caused by ST398 and of the 69 after July 2006, 22 (32%) were caused by ST398.

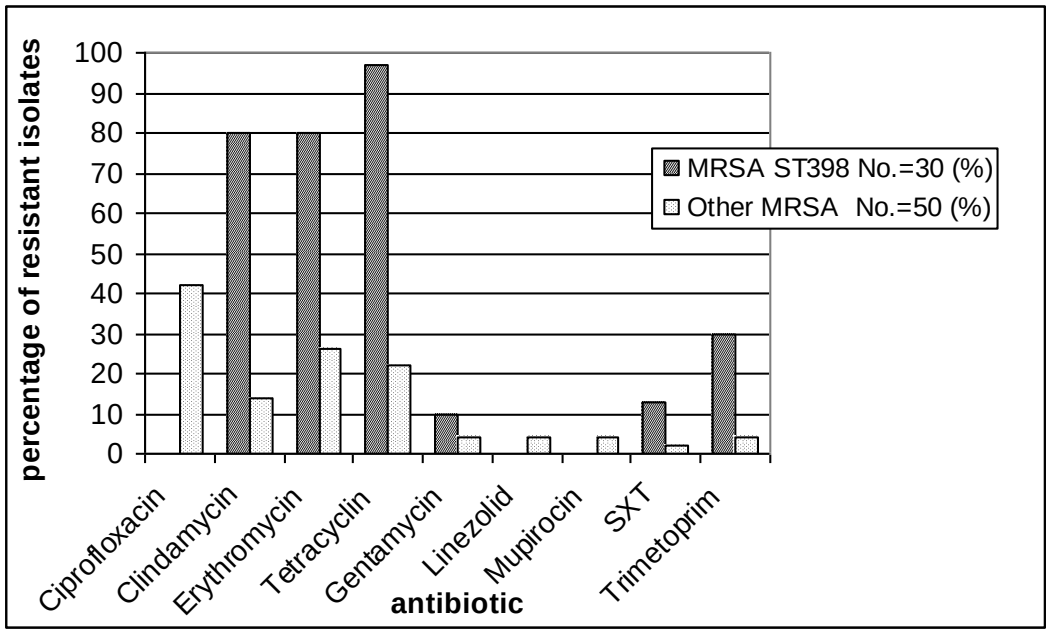
MRSA infections 2007 and 2008

Between January 1st 2007 and December 31st 2008 a total number of 416 new MRSA positive persons were identified of which 259 (62%) had MRSA ST398. Of these, a total of 80 persons had clinical samples positive of which 73 presented with an infection, 7 developed an infection later. Four patients had two periods of infection: one had a sepsis following a diabetic foot infection, one was a patient with an acute myeloid leukaemia with two episodes of pneumonia with positive sputum cultures, one patient had an urinary tract infection (UTI) after a wound infection, and one patient had two episodes of UTI. Of these patients, only the first infection was included. Table 2 shows an overview of all infections. Of the 30 patients with an MRSA ST398 infection, only 11 patients had documented contact with livestock. The three most frequent *spa*-types involved in infections with ST398 were t567 (n=11), t011 (n=9) and t108 [n=5]. A certain *spa*-type was overrepresented among the isolates that caused infections. While only 8% [18/229] of all MRSA ST398 carriers were colonized with *spa*-type t567, nearly 40% of the infections [11/30] were due this *spa*-type. No PVL positive MRSA ST398 was found. Among the 156 other MRSA isolates, a total of 21 PVL positive isolates was found of which 13 were associated with infections: 10 skin/soft tissue infections, 2 urinary tract infections and one wound infection. All these were community associated infections [CAI] without risk factors, except in one patient which was a family member of another MRSA positive patient. The associated *spa*-types were t008 (n=3), t044 (n=9) and t202 (n=1). The PVL positive isolates that were not involved in infections were t008 (n=3), t044 (n=3), t852 (n=1) and t3361 (n=1).

Susceptibility data for the clinical isolates are shown in figure 3. Of the MRSA ST398 21 (70%) isolates were resistant tot 3 or more antibiotics, whereas 5 (17%) were resistant to four or more antibiotics. Among the other MRSA isolates this was the

case in 6 (12%) and 3 (6%), respectively. Exclusively looking at the isolates that caused CAI, 13 (81%) of the MRSA ST398 isolates were resistant to 3 antibiotics versus 3 (11%) of all non-ST398 MRSA isolates.

Figure 1. Percentage of clinical isolates not susceptible to antibiotic tested.



SXT = sulfamethoxazole-trimetoprim

Table 2 Clinical samples of MRSA ST398 versus other MRSA patients.

	other MRSA [no.=50]		*MRSA ST398 [no. = 30]*	
Male	30 (60%)		21 (70%)	
Age [median]	50 (range 0-98)		56,5 (range 1-91)	
Age [average]	57.2		58.5	
No known risk group	42		18	
Livestock contact	0		11	
MRSA in family	2		0	
Other Dutch HCI	1		0	
Outbreak contact	2		1	
Community associated infections	27		16	
Health care associated infections	23		14	
PVL	13		0	
Type of clinical culture	No risk group (no.)	From known risk group (no.)	No risk group (no.)	From known risk group (no.)
Blood	4	1	0	1
diabetic foot	0	0	**4 (p<0.05)**	2
operative wound	5	2	4	2
Otitis	1	0	0	2
skin & soft tissue	**12 (p<0.05)**	1	0	0
Sputum	6	0	3	0
Urine	7	3	2	0
Wound	7	1	5	5
Total	42	8	18	12

* one patient was positive in an outbreak setting and developed an osteomyelitis

Discussion

Over the past five years there has been a vast increase of MRSA positive patients in our region. The vast majority of that increase can be explained by the change of the screening protocol, with the inclusion of patients in contact with livestock. Over 80% of all newly identified MRSA positive patients belong to this latter group. Presently, the majority of patients with ST398 MRSA are just carriers and only a single outbreak has been documented. Still, the recent emergence of ST398 MRSA is cause for concern. Of all patients with a clinical culture with MRSA, indicative of an infection with this micro-organism, one third has MRSA ST398. The spectrum of ST398 infections is not radically different from that of other MRSA with the exception of skin and soft tissue infections, which are primarily caused by CA-acquired, PVL positive MRSA. Although ST398 does not appear to be as virulent as CA-MRSA, it does cause infections in susceptible hosts and is frequently multi-resistant, making empirical antibiotic therapy in the out-patient setting difficult.

While we tried to classify MRSA infections as health care associated and community associated infections respectively, the question remains whether this classification is still applicable in the present Dutch situation. It is implied that a health care associated infection [HAI] with MRSA represents acquisition of this microorganism in the health care institution. Seen, the low prevalence of MRSA in Dutch hospitals, and the emergence of ST398 MRSA in the community, chances are that a majority of the patients acquire MRSA previous to admission to the hospital. In our hospitals, an unexpected MRSA infection is always followed by screening of health care workers and fellow patients in contact with the index. The current experience with HAI caused by MRSA ST398 shows that transmission to fellow patients and HCWs is rare [9]. Furthermore, the total number of HCW colonized with ST398 seems to be low [10], thus while the infection may start in the hospital, the acquisition of ST398 is most likely due to direct contact with livestock. Although the prevalence of MRSA ST398 in the general population appears to be low [11] acquisition through contact with contaminated environment, air or through direct contact with carriers cannot be excluded. Environmental contamination – outside "open" stables – has been

described [12] but the question remains whether this could be the case in the Netherlands where most stables are "closed". While rare case reports such as MRSA ST398 endocarditis in a woman living next to pig farm [13] and ST398 post-operative osteomyelitis after having an accident on a rural country road [within the cohort of the present study], have been described, more research is needed to establish the risk in persons with only environmental contact.

MRSA ST398 has lead to a significant increase in MRSA positive patients thereby putting a considerable strain on infection control practices in hospitals. At present the main health risk of MRSA ST398 lays with those persons in contact with livestock.

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Chapter 9: The use of Raman spectroscopy in the epidemiology of Methicillin resistant *Staphylococcus aureus* of human and animal related clonal lineages.

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Abstract

In order to perform a cost-effective Search and Destroy policy for methicillin resistant *Staphylococcus aureus*, a quick and reliable typing method is essential. In an area with a high level of animal related MRSA ST398, PFGE typing and *spa*-typing are not sufficient to discriminate between co-incidental findings and true transmission of MRSA. This study is the first to retrospectively show the performance of Raman spectroscopy in 16 well documented outbreaks. We analysed 525 isolates, 286 MRSA ST398 and 239 from other PFGE clusters with Raman spectroscopy. When epidemiologically linked isolates from the outbreaks were analysed with PFGE as a gold standard, Raman spectroscopy correctly identified 97% of cases that were indistinguishable from the index case. With Raman cluster analysis, the most dominant distinction was between MRSA ST398 and other MRSA of human clonal lineages. Within MRSA ST398, 22 different Raman clusters were identified. Raman typing correctly identified a ST398 (*spa*-type t567) outbreak in a hospital setting. No correlation was observed between Raman clusters and *spa*-types. We conclude that Raman spectroscopy is a quick and reliable method of MRSA typing which can be used in outbreak settings and it is comparable to PFGE with the added advantage that PFGE non-typeable isolates can also be readily typed using the same sample preparation protocol

Introduction

In The Netherlands the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) is low. In blood cultures positive for *S. aureus,* only 1% is methicillin resistant and carriage rates in the general population are between 0.08-0.03% [1,2]. This is thought to be due to both a restrictive use of antibiotics and an active MRSA search and destroy policy. This policy involves screening of all patients transferred from foreign hospitals, screening of all contact of MRSA positive patients including health care workers (HCW) and fellow patients and -since July 2006- people in contact with livestock (pigs and veal calves) (www.wip.nl).

In the South-East of the Netherlands (the adherence area of the PAMM laboratory), the number of newly identified MRSA positive individuals increased from 16 per year between July 2002- July 2006 to 148 per year between July 2006-Dec 2008. 81% of this increase is due to MRSA of multi locus sequence type (MLST) ST398 (Methicillin-resistantStaphylococci in Animals: Veterinary and Public Health Implications, London 2009, S3:4), a type of MRSA associated with contact with livestock. Of this 81%, the majority came from targeted screening (98 per year, 74%) but 7% was due to unexpected cases.

An unexpected MRSA case in a hospital or nursing home, will lead to a screening of close contacts of the index patient. If more MRSA positive individuals are found, an outbreak investigation will be started but the manner and magnitude of the investigation will depend on whether or not there was transmission. Consequently, the need is felt for a reliable and preferably fast typing method for all MRSA including MLST ST398 in order to make timely decisions on the outbreak investigation regime. The difficulty with MRSA of this sequence type, is that it is non-typeable with standard pulsed field gel electrophoresis (PFGE) using *Sma* I restriction [3], which was the standard method in the national reference laboratory in the Netherlands (RIVM) until 2007. As of January 2008 *spa*-typing is used but since most animal related isolates belong to a small number of closely related *spa*-types, this method does not have the discriminatory power required [4]. Raman spectroscopy is an optical method which relies on spectroscopic fingerprints that represent the

complete molecular composition of a microorganism. The method was recently shown to be effective for strain typing of staphylococci [5]. In this study we describe the use of Raman spectroscopy on isolates from screening cultures and clinical isolates of both PFGE typeable and non-typeable strains.

Materials and methods

Isolate collection

The PAMM laboratory for Medical Microbiology has an adherence area of 800.000 people in the south east of the Netherlands and provides services to four hospitals and the general practitioners and nursing homes in the area. This area also has the highest density of pig farms in the Netherlands [6]. A total of 525 MRSA positive individuals of which isolates were stored and PFGE typing data known, were identified in the laboratory database between July 2002 and December 2008. All these isolates were confirmed as MRSA by *femA*/*mecA* PCR. PFGE typing results and *spa*-types were obtained from the national reference laboratory (RIVM, Bilthoven). Of these 525 isolates, 286 were non-typeable by PFGE and/or belonged to *spa*-types corresponding with MRSA ST398.

Clinical data

Clinical data for the isolates from this study was collected from the MRSA surveillance records from the infection control departments of the hospitals, the clinical consultation form in the laboratory information system and from patient charts. For each isolate the origin was noted; clinical (unexpected MRSA) or obtained during targeted screening. For all targeted screening isolates the reason for screening was included, as well as known contacts between positive patients and/or health care workers.

Raman spectroscopy

Raman measurements and analyses were performed in blinded fashion, e.g. isolates were numbered and PFGE and *spa* typing results and clinical data were supplied only after measurements and cluster analysis was done.

For Raman measurements, all isolates were grown overnight on Trypticase Soy agar (TSA, Becton Dickinson, Franklin Lakes, NJ, USA). Samples were prepared as described previously [5]. Briefly, from a 20h culture biomass was suspended in water, transferred to a sample carrier and allowed to dry. Samples were measured on the advanced prototype of the SpectraCell*RA*® bacterial strain analyzer from River Diagnostics BV (Rotterdam, The Netherlands). For 24 samples, the system throughput time was 2 hours and this involved about 30 minutes of hands-on time.

Samples were measured over a period of 2 months. After 1 year, 45 samples were repeated together with a new batch of samples to determine the reproducibility over a longer period of time.

Data analysis

Raman types were determined using Wards cluster algorithm with a fixed cut-off established at 99.95% similarity. This cut-off was based on the lowest similarity observed between 3 full biological replicates (independent repeats from freezer stock to Raman measurement) of 116 isolates. Isolates grouped in a cluster were assigned a unique Raman type. Raman types were compared to PFGE data and a retrospective analysis was performed to see whether Raman typing data would have provided sufficient data to make adequate decisions in the context of an outbreak investigation.

Reproducibility over time was calculated as the percentage of isolates for which the replicate measurements are combined in the same cluster in the dendrogram.

Results

From a total of 525 isolates, 286 were non-typeable by PFGE (NT-MRSA). The remaining 239 isolates belonged to 52 different PFGE clusters and 40 Raman types.

Epidemiological data identified 16 potential outbreaks and/or cases of suspected transmission of typeable MRSA with a total of 142 isolates. The outbreaks occurred in nursing homes, hospitals and families. Table 1 shows the main characteristics of the outbreaks with regard to the number of isolates and correspondence between PFGE clusters and Raman types.

Of the 142 isolates, 127 (89%) cases were identified where the PFGE cluster was identical to that of the index case. In 123 (87%) cases the same applied for the Raman type. When PFGE is regarded as a gold standard, Raman spectroscopy would come to identical conclusions in 123 out of 127 cases (97%). Furthermore, in 18 cases in which the PFGE type was different from the index, the same applied with regard to Raman type (Table 1).

In retrospect, in the 6 outbreaks where PFGE showed identical types to the index case but Raman clusters were different, this would not have led to a termination of the outbreak investigation since in the same round of screening identical isolates were found as well.

Raman and NT MRSA

It was very interesting to see that the most dominant distinction between the isolates was that between PFGE typeable and NT isolates (Figure 1). This finding confirms the genetic evidence that the NT isolates are a subpopulation within the *S. aureus* species, using phenotypic data generated by Raman typing. At the first branch in the dendrogram, 512 of 525 (98%) isolates were correctly grouped in the typeable or NT cluster. Eight PFGE non-typeable isolates were designated a Raman type associated mainly with PFGE-typeable isolates. The reason could be a technical failure to produce restriction fragments. In contrast, 5 isolates were typeable by PFGE but were designated a Raman type associated with non-typeable isolates. This

resulted in a in a specificity 98% and sensitivity of 97% for distinguishing PFGE NT and ST398 isolates.

286 PFGE non-typeable isolates were divided into 22 Raman types. The three most predominant types are 30 (n=87 isolates), 26 (n=43), 28 (n=43). Seven isolates had a unique Raman type. In order to asses the clinical relevance; data were compared to known epidemiological relations between patients (Table 2). In the included hospital outbreak [7] all 9 isolates belonged to one Raman type (nr 4).

Within families exposed to livestock, different Raman types were found. This also occurred in subsequent cultures of individual patients . Recently we have shown that Raman typing was able to reliably discriminate between multiple strains colonizing a single patient [8].

It is difficult to attach significance to this finding here, since it is uncertain whether people in contact with livestock become carriers because of a one-time acquisition of the MRSA strain or because they have a continuous exposure to the source. There is some evidence for the latter since carriage of MRSA ST398 is short lived in people with a one-time exposure [9]. In addition different strains can be found if livestock is bought from different sources.

Spa typing and Raman types

For 255 of the NT isolates, *spa*-typing was available. There was no direct correlation between the two most common *spa*-types t108 and t011 and any of the Raman clusters. However, 21 of 27 isolates (77%) with *spa*-type t567 belonged to Raman type 4 and no other *spa*-types were associated with this Raman type.

Reproducibility

To asses reproducibility over time and after freezing and thawing, 20 non-typeable and 26 PFGE typed isolates were tested at two different points in time. Reproducibility was 95 % (45/46 isolates having replicates in the same cluster).

Table 1 Description of outbreak involving PFGE typeable isolates

Outbreak number	nr of isolates	PFGE types (nr. of isolates)	Raman types (nr. of isolates)	Outbreak description with comments on Raman clusters
2002-1	18	158* (n=18)	9* (n=18)	6 patients and 12 HCW[$], no discrepancies between PFGE and Raman typing
2002-2	36	158* (n= 34) 113 (n=2)	9* (n=32) 26 (n=1) 31 (n=1) 6 (n=1) 22 (n=1)	26 patients and 10 HCW[$]. Two patients with both a different PFGE and Raman from the index case. Two more discrepant Raman clusters: 26 and 31 were found in the long term follow-up of the outbreak (3 months and 9 months after initial screening started respectively)
2003-1	11	71* (n=6) 271 (n=1) 115a (n=2) 16d (n=1) 209a (n=1)	14*(n=7) 13 (n=2) 31 (n=1) 23 (n=1)	7 patients with identical Raman (14) and 6 with identical PFGE (71). Other discrepant PFGE types also had different Raman types.
2003-2	6	55*(n=4) 248 (n=2)	23* (n=4) 29 (n=2)	3 patients and one HCW[$] positive. Family members of the HCW[$] positive with both different PFGE 248 and Raman 29
2004-1	5	55*(n=2) 305 (n=1) NT (n=2)	23* (n=3) 30 (n=2)	3 family members identical Raman types but two PFGE types (55, 305) Screening of contact patients showed different Raman and PFGE types
2005-1	2	137B *(n=2)	24* (n=2)	Transmission between patient and HCW[$]
2006-1	12	15* (n=12)	31*(n=11) 33 (n=1)	2 patients and 10 HCW[$]. Discrepant Raman found in only patient (besides the index) in outbreak.
2006-2	4	113*(n=4)	11* (n=3) 10 (n=1)	Index patient and family member and HCW[$] with identical isolates. One other HCW[$] with a PFGE identical but Raman discrepant isolate. This isolate had a different resistance pattern: ciprofloxacin, clindamycin S, fusidic acid R, while the other 3 isolates had identical resistance patterns.

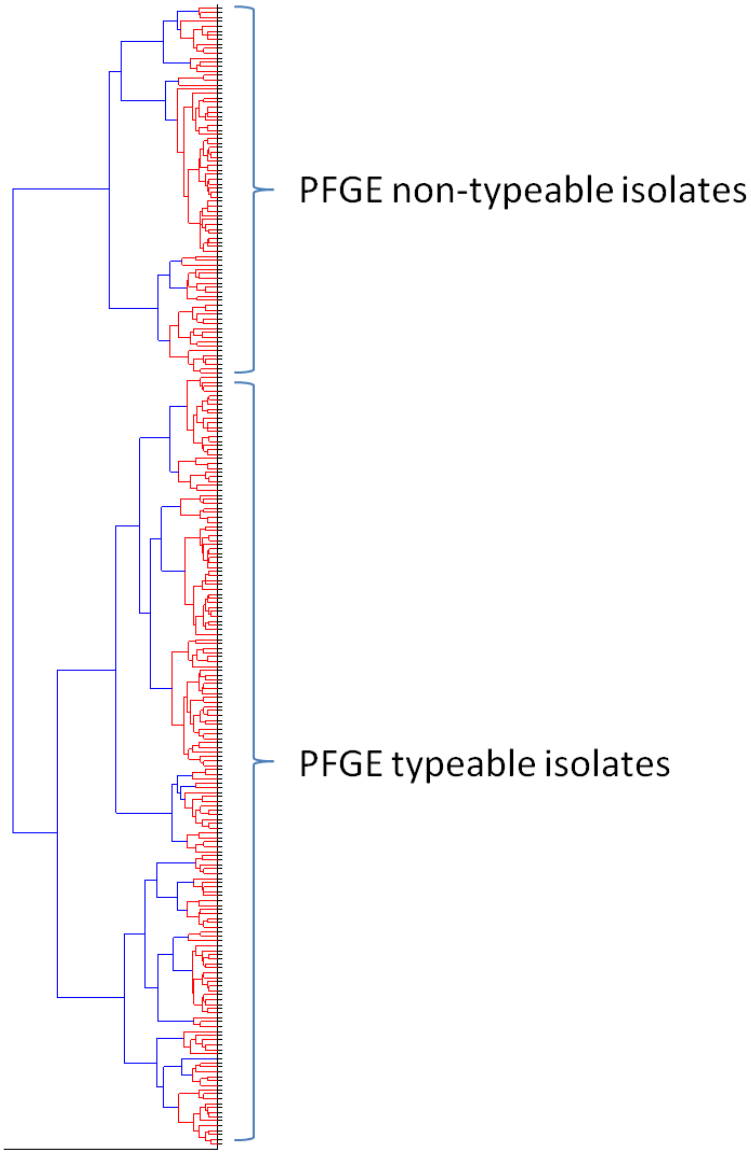
Outbreak number	**nr of isolates**	**PFGE types (nr. of isolates)**	**Raman types (nr. of isolates)**	**Outbreak description with comments on Raman clusters**
2007-2	4	113*(n=4)	10* (n=3) 28 (n=1)	One patient with a discrepant Raman cluster. Identical resistance patterns.
2007-3	3	218a* (n=2) 71a (n=1)	25* (n=2) 5 (n=1)	Family members with MRSA. One member after one month both different PFGE & Raman cluster.
2007-5	3	71a 65*(n=3)	5 17*(n=3)	Index positive (PFGE 71a, Raman 5), screening of contacts 1 HCW[$] positive with different PFGE/Raman, 2 contacts of this HCW[$] with PFGE 65, Raman 17
2007-6	2	113*(n=1) NT (n=1)	10 (n=1) 4 (n=1)	Index patient with an NT, Raman 4 isolate, HCW[$] with PFGE 113/ Raman 10. Interpreted as co-incidental finding, screening stopped after one ring.
2007-7	4	23*(n=3) NT (n=1)	27* (n=3) 30 (n=1)	Two patients and one HCW[$] with same strain, one HCW[$] with an NT isolate which also has a different Raman type (30)
2007-8	23	65*(n=23)	17* (n=23)	11 patients and 12 HCW[$] positive with identical isolates.
2007-9	6	113*(n=6)	11* (n=6)	6 patients in nursing home
2007-10	3	28*(n=3)	25* (n=3)	3 MRSA infections in one family
Total	**142**	**127****	**123****	

* = indication corresponding PFGE and Raman type to the index case

**=total number of isolates recognized as being identical to index case

[$] HCW = health care worker

Figure 1.



Dendrogram obtained from a cluster analysis on isolates in this study. The figure shows that the most dominant distinction is that between the PFGE typeable and non-typeable isolates.

Table 2 Description of outbreak involving PFGE non-typeable isolates (MSRSA ST398)

Outbreak number	Nr of Isolates (n)	*spa*-types (nr. of isolates)	Raman types (nr. of isolates)	Outbreak description with comments on Raman clusters
2006-3	2	PFGE 55 (n=1) t567 (n=1)	29* (n=2)	Transmission mother to child. Isolates with an identical resistance pattern
2007-1	2	t011* (n=1) t108 (n=1)	28 (n=1) 18 (n=1)	Contact screening around patient, one HCW[$] positive, different type of MRSA
2007-4	9	t567*(n=9)	4* (n=9)	Outbreak in hospital [7]
2007-11	5	t034*(n=4) t011 (n=1)	30*(n=2), 29 (n=1), 15 (n=1) 19 (n=1)	Family of five people living on calf farm.
2007-12	2	t108*(n=2)	30* (n=2)	Family members pig farm
2008-1	3	t011* (n=1) t567 (n=2)	12 (n=1) 4 (n=1) 20 (n=1)	Possible transmission in nursing home. Index patient with t011, 2 HCW[$]with t567, one had contact with horses but not calfs and/or pigs
2008-2	2	t011*(n=2)	28* (n=2)	2 isolates from one patient with different resistance patterns
2008-3	2	t108* (n=1) t011 (n=1)	30 (n=1) 12 (n=1)	2 isolates from one patient with different resistance patterns
2008-4	2	t011*(n=2)	18 (n=1) 26 (n=1)	2 isolates from one patient with different resistance patterns
Total	**28**	**20**	**17**	

* = indication of *spa*- and Raman type corresponding to the index case

**=total number of isolates recognized as being identical to index case

$ HCW = health care worker

Discussion

In the context of an active search and destroy policy, rapid MRSA typing methods are important. When an MRSA is found in a patient, a first ring of contacts including patients and health care workers is screened (direct contacts of the index case). If other individuals are found carrying the same strain, the net is thrown wider and a second or even a third ring is screened (indirect contact with index). If the other MRSA isolates belong to a different strain (e.g. have a different PFGE or Raman type), a limited circle of people will be screened around this new index. Therefore a rapid and reliable typing method is essential to distinguish between transmission and a co-incidental finding and to limit the screening efforts and associated costs. In this study Raman typing had a 97% similarity compared to typing by PFGE. The advantage of Raman spectroscopy is that it is a fast method and less labour intensive than PFGE, a significant advantage in outbreak settings. Reproducibility of Raman results over a period of one year was good; 95 %(45/46 isolates showing replicate samples in same Raman cluster).

Furthermore, Raman typing was able to divide so-called non-typeable MRSA into 22 distinct types. Raman typing correctly identified a ST398 (*spa*-type t567) outbreak in a hospital setting. The different types seen in families could be due to the either high discriminatory power of the Raman method or might mirror the fact that families harbour different types of MRSA due to the exposure to a common source (livestock) that might harbour different types of MRSA. The fact that there is a lack of clear correlation between Raman and *spa*-typing can be explained by the fact that a number of the closely related *spa*-types are found within the NT MRSA and *spa*-typing appears not to have a high enough discriminatory value for epidemiology within ST398. , which is not unexpected since *spa*-typing involves repeats in one gene.

In order to type ST398 strains, modified PFGE procedures have been documented as wel as typing by MLVA [4, 10]. It was shown that different *spa*-types can occur within the same MLVA cluster and vice versa [4]. It would be interesting to compare these methods to Raman typing.

Conclusions

Raman spectroscopy is a quick and reliable method of MRSA typing which can be used in outbreak settings and it is comparable to PFGE with the added advantage that PFGE non-typeable isolates can also be readily typed using the same sample preparation protocol. Isolates with a correlation coefficient of 99.95% or higher should be considered identical, but more research is needed to establish which isolates should be considered closely related.

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Chapter 10: Comparison of the BD GeneOhm™ MRSA and Cepheid Xpert™ MRSA Assays in a region with a high prevalence of livestock associated Methicillin resistant *Staphylococcus aureus* ST398: Difficulties in detecting certain variants with PCR but not by chromogenic agars.

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Abstract

In a country with a low MRSA prevalence and an active search and destroy policy, rapid diagnostic tests have the potential benefit of preventing isolation days for patients. Although most commercial assays have a high sensitivity and specificity in general, certain types of MRSA might cause problems. In the south-east region of The Netherlands, the majority of MRSA positive people carry livestock-associated MRSA (LA-MRSA) strains. Two commercial rapid molecular diagnostic tests, the BD GeneOhm™ MRSA assay and the Cepheid Xpert™ MRSA assay, were tested on isolates of locally prevalent MRSA types, consisting of 30 non-LA-MRSA and 40 LA-MRSA isolates, and in addition on an international collection of 16 LA-MRSA isolates The PCR methods were compared with a chromogenic culture medium (chromID MRSA agar). Both PCR assays detected 93% (28/30) of non-LA-MRSA but detected only 68% (27/40) of local LA-MRSA. These findings can be explained in part by the presence of a local clone of *spa*-type t567 harbouring SCC*mec* V, which is not a very common *spa*-type in the Netherlands but has a relatively high prevalence in our region. In the international collection of 16 LA-MRSA, 11 (69%) and 13 (81%) were positive in the BDGO assay and Xpert™ MRSA assays, respectively. When implementing MRSA PCR assays these should therefore be evaluated in the context of the local MRSA epidemiology.

Introduction

In 2006, contact with livestock was added to the Dutch search and destroy guidelines as a risk factor for carriage of methicillin-resistant *Staphylococcus aureus* (MRSA). People in contact with livestock often carry isolates of a distinct MRSA lineage which is non-typable by PFGE with *Sma*I (16,18) and consists of closely related *spa*-types (mainly t011, t034, t567, t571) corresponding to clonal complex (CC) 398 by multilocus sequence typing (MLST) (16, 18, 19, 20).

The adherence area of the PAMM Laboratory for Medical Microbiology consists of the city of Eindhoven and a rural area with a high density of pig farms. In this catchment area of approximately 800.000 people in the south-east of The Netherlands, the implementation of screening of people in contact with livestock led to an increase in detection of MRSA carriers from an average of 20 per year between 2002-2005 to 145 per year between 2006-2008; 82% of this increase was caused by MRSA CC398 (21). Of the 356 MRSA-positive individuals identified by targeted screening in 2007-2008, 241 (67%) carried an MRSA CC398 (21). The increase in MRSA CC398 carriage rates could lead to an influx into hospitals of individuals who harbour MRSA (17,21). With the prevailing Dutch search and destroy guidelines this leads to a significant increase in patients that have to be placed in preempitive isolation. Because the standard culture method is time-consuming, there is an urgent need for a fast and sensitive diagnostic tool to identify MRSA carriers and especially non-carriers. Two commercially available PCR assays, the BD GeneOhm™ (BDGO) MRSA assay (BD Diagnostics GeneOhm, Québec, Canada) and the Cepheid Xpert™ MRSA assay 9 (Cepheid Xpert™ MRSA, Sunnyvale CA), both designed to detect MRSA from human nasal swab specimens, were evaluated tested on isolates representing the specific epidemiological profile of our setting and on representative isolates from an international collection of MRSA CC398 and compared to the bioMérieux chromID MRSA agar.

Materials and Methods

Isolates

The strain collection consisted of 86 isolates: 30 MRSA representing the most frequent PFGE types found in our region between 2002 and 2007 (collection A); 40 consecutive MRSA isolates that were non-typable by PFGE using *Sma*I (collection B) and 16 MRSA CC398 from attendees at a pig health conference in Denmark, 2006 (collection C) which were characterized by PFGE, *spa* and SCC*mec* typing in previous studies (10,20). All isolates were confirmed as MRSA by a *mecA*/*femA* PCR as described elsewhere (14).

BD GeneOhm™ MRSA assay

The BD GeneOhm™ (BDGO) MRSA assay (BD Diagnostics GeneOhm, Québec, Canada) is developed for testing on nasal swabs so an adjusted protocol was used for testing on pure cultures. Colonies were suspended in saline to 0.5 McFarland (1 x 10^8 CFU/mL). A swab was dipped in this suspension and transferred to the lysis tube, which was then processed as recommended by the manufacturer's instructions with exception of time of the heating step and the instrument used for the real-time PCR amplification step. Briefly, the lysis tubes containing the cell suspension were vortexed for 5 minutes at high speed. To remove the cellular debris and unlysed cells, the samples were centrifuged briefly (quick spin) at low speed and heated at 94°C for 10 minutes. The lysed samples were kept on ice or cooling block until further use. After centrifugation of the bacterial residues, 2.8 µl of lysed sample was added to the BDGO reconstituted Master mix (25 µL) and subsequently analysed on the AbiPrism7000.

Cepheid Xpert™ MRSA assay

The Cepheid Xpert™MRSA assay is designed for use on nasal swabs so an adjusted protocol was used for testing the isolates. A bacterial suspension of 0.5 McFarland was diluted 1:1000 in PBS according to manufacturers instructions and 75 µl was added to the Xpert™ MRSA elution reagent and subsequently analysed in the Xpert™ MRSA assay according to the manufacturer's instructions.

Chromogenic agar

From all isolates, 10 µl of a 0.5 McFarland suspension was cultured on the chromID MRSA agar (bioMérieux, Baumes les Grottes, France) and judged after 18 h for the growth of green colonies.

Molecular typing

All 86 MRSA isolates were genotyped as follows. Pulsed-field gel electrophoresis (PFGE) using *Sma*I was performed as described elsewhere (12). Isolates were further characterised by *spa* typing according to the RIDOM protocol (5). The *spa*-types were assigned to the corresponding CCs based on the *spa*-type association to known MLST types by using the Ridom database (www.ridom.de). The structural features unique to each of the 6 major allotypes of the SCC*mec* element, types I–VI, were determined by a multiplex PCR (M-PCR) assay described by Kondo et al. (9): *ccr* types 1-5 (M-PCR 1), *mec* classes A, B, and C2 (M-PCR 2), and the J1 regions of four subtypes (a-d) of type IV SCC*mec* (M-PCR 3). The class C1 *mec* gene complex was determined by conventional PCR using primer mA7 (9) in combination with primer IS2-L (15) under the same conditions as for M-PCR 2. MRSA strains JCSC6082 and JCSC6945 carrying SCC*mec* types VII (5C1) and X (6C1), respectively, were used as reference strains (1,10). The sizes of the DNA fragments estimated from the nucleotide sequences are 1,266 and 306 bp for *mecA*-IS*431*, in agreement with the fact that IS*431* is inserted 968 bp and 17 bp downstream of the *mecRI* start codon in JCSC6082 (archetypical C1) and JCSC6945 (C1.2), respectively (10). SCC*mec* nomenclature was as proposed by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (8). For brevity, the type is indicated by Roman numerals, the subtype, identified by a lower-case Latin letter. The *ccr* and *mec* gene complexes are indicated by an Arabic number and Latin letter, respectively, in parentheses.

Results

Assay results

All 86 MRSA isolates grew with green colonies after 18 h on the chromID MRSA agar. The results of the molecular assays are shown in table 1. Of the 32 typeable (non-CC398) MRSA isolates (collection A), 28 (93%) were positive in both the BDGO assay and in the Xpert™ MRSA assay. The 2 negative isolates carried SCC*mec* type IVc and SCC*mec* type IVd/SCC476 and belonged to *spa*-types t535 and t447 and PFGE types 22 and 55, respectively.

Of the 40 MRSA CC398 from collection B, 27 (68%) were positive in both the BDGO assay and in the Xpert™ MRSA assay and 13 were negative in both essays. The majority (85%; 11/13) of false-negatives belonged to *spa*-type t567 and carried SCC*mec* V (n=9) and SCC*mec* VII (n=2). The remaining 2 false-negatives belonged to *spa*-type t108 and carried SCC*mec* V.

Of the 16 MRSA CC398 from collection C, 11 (69%) and 13 (81%) were positive in the BDGO assay and Xpert™ MRSA assays, respectively. Among the false-negatives, three were negative in both assays (two t034 isolates carrying SCC*mec* V and X, respectively, and one t567 isolate carrying SCC*mec* V) and two were negative in the BDGO assay but positive in the Xpert™ MRSA assay (one t011 isolate carrying SCC*mec* V and one t034 isolate carrying SCC*mec* IX).

Molecular typing

The *spa*-types and SCC*mec* types of all 86 MRSA isolates are depicted in table 1.

Of note, a class C1-like *mec* gene complex identical to that in SCC*mec* X of MRSA CC398 strain JCSC6945 (10) was identified in two t567 isolates (collection B) in combination with a type 5 *ccr* gene complex. Consequently, the SCC*mec* was classified as type VII (5C1). A fusidic acid-resistant t447 isolate belonging to PFGE 55 carried a combination of types 1 and 2 *ccr* gene complexes and a class B *mec* gene complex. SCC*mec* subtyping showed that it harboured SCC*mec* IVd (2B).

Table 1. Results of MRSA assays and SCC*mec* typing data.

PFGE type	spa*-type*	*SCC*mec	ccr	*mec*	*No.*	*BDGO*		*Xpert MRSA*	
						Pos	**Neg**	**Pos**	**Neg**
Collection A									
15	t005	IV	2	B	1	1	0	1	0
	t032	IV	2	B	1	1	0	1	0
22	t535	IVc	2	B	1	0	1	0	1
	t1437	IVc	2	B	1	1	0	1	0
23	t008	IV	2	B	2	2	0	2	0
49	t012	IV	2	B	2	2	0	2	0
55	t002	II	2	A	1	1	0	1	0
	t447	IVd[c]	2	B	1	0	1	0	1
65	t437	IV	2	B	1	1	0	1	0
	t2855	IV)	2	B	1	1	0	1	0
71	t037	III	3	A	2	2	0	2	0
113	t015	IV	2	B	1	1	0	1	0
	t038	IV	2	B	1	1	0	1	0
137	t008	IV	2	B	2	2	0	2	0
158	t022	IV	2	B	1	1	0	1	0
	t790	IV	2	B	1	1	0	1	0
281	t032	IV	2	B	1	1	0	1	0

PFGE type	**spa-*type***	***SCC*mec**	**ccr**	***mec***	***No.***	***BDGO***		***Xpert MRSA***	
						Pos	**Neg**	**Pos**	**Neg**
	t037	IV	2	B	1	1	0	1	0
305	t067	IV	2	B	1	1	0	1	0
	t1437	IV	2	B	1	1	0	1	0
137b	t197	IV	2	B	1	1	0	1	0
	t790	IV	2	B	1	1	0	1	0
218a	t008	IV	2	B	4	4	0	4	0
Collection B									
NT[a]	t011	IV	2	B	2	2	0	2	0
	t011	V	5	C2	9	9	0	9	0
	t034	IV	2	B	2	2	0	2	0
	t034	V	5	C2	1	1	0	1	0
	t108	V	5	C2	13	11	2	11	2
	t567	V	5	C2	9	0	9	0	9
	t567	VII	5	C1-like	2	0	2	0	2
	t571	V	5	C2	1	1	0	1	0
	t4283	IV	2	B	1	1	0	1	0

PFGE type	**spa*-type***	***SCC*mec**	**ccr**	***mec***	***No.***	***BDGO***		***Xpert MRSA***	
						Pos	**Neg**	**Pos**	**Neg**
Collection C									
NT[a]	t011	IV	2	B[d]	3	3	0	3	0
	t011	V	5	C2	4	3	1	4	0
	t034	V	5	C2	1	0	1	0	1
	t034	IX	1	C2	1	0	1	1	0
	t034	X	6	C1-like	1	0	1	0	1
	t108	V	5	C2	1	1	0	1	0
	t567	V	5	C2	1	0	1	0	1
	t571	V	5	C2	1	1	0	1	0
	t899	IV	2	B	1	1	0	1	0
	t899	V	5	C2	2	2	0	2	0

[a] NT; non-typeable by pulsed-field gel electrophoresis (PFGE).

[b] Isolates from strain collection C have been characterised by PFGE, *spa* and SCC*mec* typing as described elsewhere (10,20).

[c] This isolate also carries SCC476.

[d] 1 isolate contained a larger-than-normal class B *mec* gene complex (4,159-bp amplicon) due to insertion of a free copy of IS256 45 bp downstream of the *mecRI* start codon on the opposite strand (10)

Discussion

There were some deviations between our protocol and that recommended by BD: we used an Abiprism 7000 instead of a Light cycler and samples were heated during 10 minutes instead of 2 minutes. However, isolates were sent to BD where similar results were obtained.

On the basis of the tested MRSA isolates, the BDGO assay had a sensitivity of 78% and the Xpert™ MRSA assay of 80% on the strain level. Both assays detected 28 of the 30 non-CC398 MRSA (sensitivity 93%) but failed to detect 13 of the 40 local MRSA CC398 resulting in a sensitivity 68%. In the context of an active MRSA search and destroy policy to prevent unnecessary isolation days, this sensitivity is unacceptable in our region were 60-70% of all new MRSA carriers have an MRSA of this type. The question is whether this is a regional problem or also applicable to other locations with a high prevalence of MRSA CC398. In favour of a regional problem is the fact that PCR false-negatives are mostly explained by the presence of a local clone of t567/V, which is not a very common *spa*-type among MRSA CC398 (7) in general but is specifically higher in the south-east region of The Netherlands (21). In fact, even the false negative t567 from collection C was from a Dutch veterinarian.

Spa-type t567 was furthermore found to be limited to farms belonging to one supply chain (3) which is in favour of a clonal spread this *spa*-type t567 with a type of SCC*mec* cassette that is not detected by either commercial PCR tested. However, 2 out of 13 MRSA of *spa*-type t108 and 1 of 6 t034 were also missed in both assays, which are much more prevalent *spa*-types.

Both commercial assays target the *orfX* gene of the *S. aureus* core genome and a number of targets in the J3 region of the SCC*mec* cassette, which is located between *orfX* and the *mec* gene complex, and not the *mecA* gene itself. The J3 regions of type V SCC*mec* are structurally heterogeneous. The type V(5C2) SCC*mec* element of CA-MRSA strain WIS contains a 1.8-kb J3 region consisting of a single ORF (subtype 1), whereas the 10-kb J3 region in the type V (5C2&5) SCC*mec* element of CA-MRSA strains TSGH17, PM1, and JCSC5952 consists of a SCC carrying *ccrC1* allele 2. The J3

region of the predominant type V(5C2&5) SCC*mec* element identified in a collection of European LA-MRSA strains showed 100% nucleotide identity to those of SCC*mec*TSGH17 and SCC*mec*PM1 (10). Of note, Boyle-Vavra and Daum yielded positive results for MRSA ST59 strains carrying the type V (5C2&5) SCC*mec* element (2). Accordingly, the majority of type V (5C2&5) SCC*mec*-bearing LA-MRSA isolates were positive in both assays in this study. On the other hand, novel J3 regions have been identified in type V(5C2)-bearing LA-MRSA strains from Canada (4) as well as in type IX and X SCC*mec* elements carried by LA-MRSA strains from Thailand and Canada, respectively (10). It is therefore possible that the local clone t567-V also contains a novel J3 region and that the false-negative results are due to absence of target sequences or, alternatively, that the primers and/or probe are located too far from each other due to integration of novel genetic elements. Another explanation could be that point mutations in the J3 region or in *orfX* render the primers or probe unable to hybridize to the target DNA. A recent study by Reischl et al (13) showed there were novel single nucleotide polymorphisms (SNP) in the SCC*mec-orfX* integration site in LA-MRSA, also causing false negative results in both the GeneXpert and the BD GeneOhm assay. In their study 5 isolates had a non-typable SCC*mec* and one isolate carried a SCC*mec* IVa and one a SCC*mec* VII whereas the majority (78%, 14/18) of our false-negative MRSA CC398 isolates harboured SCC*mec* V. It has recently been shown that this SCC*mec* V element is a composite of an SCC carrying *ccrC1* allele 2 and a type V SCC*mec* carrying *ccrC1* allele 8 and class C2 *mec* gene complex (10).

The other false-negative MRSA CC398 isolates carried rare types i.e. VII, IX, and X. For both SCC*mec* IX and SCCmec X the J3 regions are highly diverse from other known SCC*mec* elements (10). The sequence of the type VII SCC*mec* element identified in this study is currently unknown. It would be interesting to see whether these elements have SNP's similar to those found by Reischl et al. (13)

The false-negative fusidic acid-resistant t447 isolate belonging to PFGE 55 carried a combination of types 1 and 2 *ccr* gene complexes and a class B *mec* gene complex. SCC*mec* subtyping showed that it harbours SCC*mec* IVd (2B). In the genome

sequence of the fusidic acid-resistant strain *S. aureus* MSSA476, the genetic determinant of fusidic acid resistance, *fusC*, is located inside the staphylococcal chromosome cassette SCC476, which harbours *ccr1*-like sequences (6). The t447 isolate from our collection was shown to carry *fusC* by use of a PCR-based multiplex assay (11). These findings support that it harbours both a type IV SCC*mec* (2B) and SCC476 (data not shown). Given that the SCC476 element is integrated at the 3' end of *orfX* in MSSA476, it is tempting to speculate that SCC476 is located between *orfX* and SCC*mec* IVd (i.e., in the J3 region) in the t447 isolate. If this is the case, the primers and/or probe could be located too far from each other, which would in turn provide an explanation of the false-negative results.

As was shown by the results of the chromID MRSA agar, in areas with prevailing livestock MRSA, culture methods appears to be more robust in the detection of MRSA than current commercially available molecular assays at the strain level. These data do not address sensitivity at patient level where inoculum and growth of other bacteria can influence both the performance of PCR based assays but also for chromID agars. In addition some rarely isolated methicillin-resistant staphylococci (*Staphylococcus sciuri*, *Staphylococcus hyicus*) also grow on these agars, but this constitutes only a minor drawback. Although in the Dutch situation, the longer time to detection in a culture based method may lead to unnecessary isolations precautions, the costs of these should be weighed against the cost of the faster diagnostic assays and their diagnostic accuracy in specific circumstances.

We conclude that molecular MRSA assays, in this case the BDGO assay and the Xpert™ MRSA assay, should be evaluated against the local MRSA epidemiology especially in area's with high local prevalence of MRSA CC398 because of the high diversity of SCC*mec* elements in this clonal lineage.

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Chapter 11: General discussion and summary

Aim of this thesis was to investigate the prevalence of MRSA carriage in people in contact with livestock and how this affects the risk of MRSA carriage and infection of humans in the community and in health care settings. Furthermore, we investigated the application of existing and new diagnostic methods for this emerging MRSA clone.

In the Netherlands a successful MRSA search and destroy policy has been in place since the 1980's which has kept hospital associated MRSA infections at a very low level. However, recently community associated MRSA infections have been increasing. In this thesis, we established that contact with livestock as a risk factor for acquisition of MRSA. In **chapter 2** the accidental discovery of MRSA carriers with no known risk factors for MRSA but with a possible link between MRSA carriage and contact with pigs is described. A follow-up survey showed that the prevalence of MRSA carriage among pig farmers was hundreds of times higher than that of other Dutchman and that all MRSA carried by pig farmers had specific characteristics: they were non-typable with pulsed field gel electrophoresis with *Sma*I, the reference typing method used by the Dutch national reference laboratory (RIVM) at that moment. Spa typing showed that they belonged to a number of closely related *spa*-types.

By using the Ridom database (www.ridom.de), these *spa*-types can all be assigned to of Multi Locus Sequence Typing (MLST) 398. The fact that this MRSA was non-typable by PFGE with *Sma*I was due to a novel DNA methylation enzyme present in these isolates [1].

In order to asses how widespread the dissemination of this unusual type of MRSA was, we looked for a group of people that had contact with a broad range of (livestock-)animals all over the Netherlands. This resulted in a study to estimate the carriage-rate of MRSA among Dutch farm-animal veterinarians and Dutch veterinary students. The results of this study are shown in **chapter 3.** In 2005 veterinarians and veterinary students had a much higher prevalence of MRSA carriage than

patients admitted to hospitals (4.7 versus 0.03%) [2]. Actually this prevalence was comparable to the one seen in patients admitted to Dutch hospitals from foreign hospitals (4.7% and 5%, respectively) [3]. This established that the "pig-MRSA" was not only a local but national problem.

A case-control study performed by van Loo et al [4] showed contact with pigs and veal calves as a major risk factor. The performed studies lead to an amendment in the Dutch search and destroy policy in July 2006 which stated that patients who come into contact with live pigs or veal calves have to be isolated and screened on admission to a hospital.

In order to get a first impression whether this was a Dutch or an international problem, we went to an international conference on pig health (IPVS, Copenhagen) and asked attendees to participate in a prevalence study. The results of this survey are described in **chapter 4**. It clearly shows that MRSA ST398 was widespread, with participants from The Netherlands, Germany, Spain, Belgium, Canada, Thailand and Italy carrying 'pig-related' MRSA strains belonging to MLST398. Among participants, there was a clear association between the time spent in livestock stables and the risk of being MRSA positive. The use of personal protective equipment such as gowns and gloves and masks did not protect against acquisition of MRSA. Counterintuitive was the fact that statistically, not wearing a mask was even protective with an OR of 0.38 (CI 0.12 to 0.99). However, in stables most masks are used as protection against dust particles. They are often reused and could therefore become a source of MRSA. Just like in the Dutch prevalence study, all MLST 398 isolates from this study were tetracycline resistant, but 53% of the isolates found in this study were truly multi-resistant in the sense that they were resistant to five or more classes of antibiotics. The use of antibiotics in farming could play a role in the selection of MRSA. When we looked at organic farming, the carriage rate of MRSA in farmers appeared to be lower (**chapter 5**). Further investigations by other research groups showed that there is a relationship between the use of antibiotics on a farm and MRSA carriage in humans and animals both [5].

Changing the search and destroy policy: impact on health care

The amendment in the search and destroy policy immediately led to the question what to do with healthcare workers (HCWs) living on fams with pigs or veal calves. According to Dutch guidelines all HCWs at risk for MRSA colonization (e.g. after working in hospital in a foreign country) should be screened before they start to work in a Dutch hospital and all MRSA positive HCW are excluded from working in direct patient care. To assess the magnitude of LA-MRSA among HCWs, we did a surveillance study in one university and four general hospitals in the south of The Netherlands, an area with a high density of pig farms (**chapter 6**). In these hospitals, 4.4% of HCWs had contact with either pigs or veal calves. Among these MRSA carrriage rate was low (one carrier found, 1.3%) and not statistically significant different from that in HCWs without animal contact. All in all this study supports the decision not to include HCWs in contact with pigs and veal calves in the search and destroy policy, in the absence of nosocomial spread. However, incidents involving HCW colonised with MRSA ST398 may happen. **Chapter 7** describes the first outbreak of MRSA ST398 in a Dutch hospital. In this example, none of the patients had contact with livestock but one of the HCW lived on the premises of a pig farm although she did not come into direct contact with the animals.

MRSA ST398 carriage by a HCW is a rare occurrence in daily practice. However, when it does occur, a medical-ethical dilemma arises. According to the Dutch guidelines, an MRSA positive HCW is not allowed to work in direct patient care, irrespective of why the HCW was screened. Although there is no active screening policy for HCW, at times, we "accidentally" find MRSA-positive HCWs in the absence of nosocomial spread, e.g. when the HCW becomes a patient or is screened due to other reasons. All known MRSA-positive HCWs will undergo active MRSA-decolonization treatment

An ethical dilemma arises, if the HCW's decolonization fails, due to the fact that he or she is continuously exposed to the source. Why does a HCW who was found positive "at random" and did not contribute to nosocomial spread of "their" MRSA needs to stop working in patient-care, while colleagues with comparable risk-factors

for MRSA-carriage are not even screened. Consequently the present guideline is seen as unjust and inconsequent with regard to HCW-screening. An active search policy among HCWs could lead to loss of able and motivated staff, a much more detrimental effect on health care quality than the presence of a limited number of LA-MRSA carriers, especially since MRSA ST398 does not seem to spread as easily as epidemic MRSA clones [6]. Close monitoring of this situation is warranted, especially if cases of transmission occur on a more frequent basis than thus far perceived. **Chapter 8** describes the impact of MRSA ST398 on the resources needed for screening and isolation of patients and on the number of MRSA infections in the South-East of The Netherlands. In this region the number of new MRSA positive patients increased from 16/year between July 2002-July 2006 to 148/year between July 2006-July2008. 82% of this increase came from MRSA ST398. From the 80 MRSA infections documented in 2007-2008, 30 (38%) were caused by MRSA ST398 and only 11 (36%) of these patients had known contact with liverstock. This illustrates the high demand that this MRSA makes on infection control resources.

The aim of the search and destroy policy is to prevent both MRSA transmission and healthcare-associated MRSA infections. Most of the costs of the preventive measures are linked to cultures, isolation precautions, and the labour of HCW and infection control nurses. LA-MRSA threatens the compliance with the Dutch search and destroy policy especially in those area's with a high density of pig farms. Hospitals in regions with many livestock farms are in an unfavourable position with regard to costs of this policy when compared to those in non-rural areas. They not only have to deal with a higher number of new positive patients but these patients or health care workers go back to the source and are continuously exposed, resulting in a cumulative effect on screening and isolation procedures. Still, the real "price" of isolation is paid by the patient, since isolation precautions can result in a lower standard of care [7].

The fact that MRSA emerged in livestock and is transmitted to humans cannot be seen out of the context of the use of antibiotics in these animals. This is illustrated by the fact that in this case series 70% of MRSA ST398 were resistant to 3 or more

antibiotic classes, and 17% were resistant to four or more antibiotic classes. Molecular analysis of ST398 has thus far lead to the detection of many resistance genes, such as *tet*(M), *tet*(K) and *tet*(L) that convey resistance to tetracyclins, *erm*A, B, C and T causing resistance to clindamycine and erythromycin, *vg*A and *vg*C (lincosamide resistance) *dfr*K (trimetoprim resistance), aacA-aphD, aphA3, aadD and spc (aminoglycoside resistance) and many more [8-10]. This multi-resistant phenotype is in sharp contrast to that of community associated MRSA.
To investigate nosocomial transmission of MRSA ST398, a quick and reliable typing method is needed. PFGE typing with *Sma*I is not possible and PFGE typing with other enzymes such as *Xma*I can be done but is labour intensive and *spa*-typing does not have enough discriminatory value [11]. **Chapter 9** describes the usefulness of Raman spectroscopy for typing MRSA including ST398. Raman spectroscopy relies on inelastic scattering, or Raman scattering, of monochromatic light, usually from a laser. When the bacterial strain is hit with the laserlight, spectroscopic fingerprints are created that represent the complete molecular composition of a microorganism.

By analysing an historical collection of 286 MRSA ST398 isolates and 239 isolates from other PFGE clusters, it was demonstrated that for the human clonal lineages this technique was comparable to PFGE in identifying outbreaks. Furthermore MRSA ST398 could clearly be distinguished from other, human MRSA. A MRSA ST398 outbreak was also correctly identified and Raman typing was able to distinguish 22 subtypes in MRSA ST 398. This also implies that ST398 has a highly divers molecular composition.

This was also demonstrated in **chapter 10** where commercial PCR assays used for the routine detection of MRSA carriers, failed to detect 20% of MRSA ST398 isolates. Further analysis revealed that MRSA ST398 carried unusual and new types of SCC*mec* cassettes of which the place of origin needs to be determined. It was established by the group of Ito et al that they were distinct from those normally found in humans [12]. It could be speculated that the transfer of SCC*mec* cassettes from other staphylococci occurs more easily in the animal environment with antibiotic pressure as a facilitating factor. In addition to this, genetic elements have

been found in the SCC*mec* cassette of ST398 strains that are related to the detoxification of heavy metals such as copper and zinc [12]. These metals are used as supplements in animal feeds in some countries and provide another selective pressure in favour of MRSA ST398 besides antibiotics.

Concluding remarks

MRSA ST398, the main clonal complex (CC) associated with livestock, has been found in various animals and humans all over the world and by now transmission has been described amongst and between different kind of animals including humans. Pigs, farmers and veterinarians in Canada, Belgium, Italy, Germany the USA and Denmark are found to be carriers of this MRSA clone [13-18]. Data about veal calves show similar levels of colonization [19]. MRSA ST398 has spread in an animal hospital, causing infections such as catheter associated infections and post-operative wound infections in horses, similar to those of HA-MRSA in humans [20]. In an article by Yu et al, ST398 is named as a cause of hospital associated infections in China [21] and a lethal ventilator associated pneumonia was described in Italy [22].

On the other hand MRSA ST 398 does not appear to spread effectively in the general population and is mainly limited to those in contact with the animals. [23]. Although isolates with certain virulence factors, such as PVL have been found and serious infections have been described [24,25] the CC appears to be more like the HA-MRSA of old: in general not very virulent, but capable of causing infections in people with underlying disease or people with defects in the skin barrier.

Whether MRSA ST398 is 'here to stay' within the human population remains to be seen, but it has shown the potential to both colonise and infect humans and a broad range of other animals and has acquired resistance to many antibiotics. Unfortunately, there is no magic wand to make this MRSA disappear, in normal or in 'alternative' medicine [26]. The routine use of antibiotics, which disrupts the normal bowel flora in piglets, could be a selective pressure and thus a contributory factor in the occurrence of ST398 in these animals. A concerted effort from both human and veterinary medicine is needed to prevent further spread. In the meantime, MRSA

ST398 should be considered an occupational health risk in people in contact with livestock.

In ancient Greece it was considered a basic fact that there was a link between infections in animals and in humans. This was long forgotten, until it became clear in the 19th century that micro-organisms such as *Mycobacterium tuberculosis* and *Bacillus anthracis* could transfer disease from animals to humans. More recently, the large Q fever outbreak in the Netherlands brought this message forcefully home again. Although the number of people with infections from livestock associated MRSA is still low, the emergence of this clone has to be seen in the context of other multi-resistant organisms that can be transferred from other animals to humans. Examples are vancomycine resistant enterococci, multi-drug resistant *Salmonellae* and – even more recently- ESBL carrying Shiga-toxine producing *E. coli*. All of these are a clear warning that antibiotics should be 'handled with care' in humans and other animals alike. Their use should be for the treatment of bacterial infections only. Antibiotics are a too valuable commodity to waste. If we do not take care, we might be returning to a pre-antibiotic era within decades.

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Chapter 12: Samenvatting en conclusies (Nederlands)

Methicilline resistente *Staphylococcus aureus* was van oorsprong een 'ziekenhuisbacterie' maar wordt in toenemende mate ook buiten het ziekenhuis aangetroffen. In dit proefschrift wordt onderzoek beschreven naar de prevalentie van MRSA dragerschap in mensen in contact met vee en naar de invloed die dit heeft op MRSA dragerschap en infecties in en buiten het ziekenhuis. Daarnaast onderzochten wij de toepassing van nieuwe en bestaande diagnostische technieken op de vee gerelateerde MRSA.

Nederland kent al sinds de jaren '80 van de vorige eeuw een succesvol MRSA 'search and destroy' beleid waardoor het aantal ziekenhuisinfecties met MRSA zeer klein is. Recent neemt echter het aantal mensen met MRSA dragerschap en/of infecties buiten het ziekenhuis toe. In dit proefschrift stellen wij vast dat contact met vee een risicofactor is om MRSA positief te worden. In **hoofdstuk 2** wordt beschreven hoe enkele onverwacht MRSA positieve patiënten, dat wil zeggen patiënten zonder bekende risicofactoren, aanleiding gaven tot verder onderzoek. Deze patiënten bleken allen contact met varkens te hebben. Een vervolgonderzoek onder varkenshouders uit de regio toonde aan dat de prevalentie van MRSA dragerschap onder deze beroepsgroep honderden keren hoger was dan onder de algemene bevolking. Alle geïsoleerde MRSA stammen van deze varkenshouders hadden gemeen dat zij niet typeerbaar waren met PFGE met *Sma1* (de standaard typeringsmethode van het RIVM op dat moment) en dat zij tot een nauw verwante groep van *spa*-types behoorden. Met behulp van de Ridom database (www.ridom.de) kon worden aangetoond dat al deze *spa*-types behoren tot Multi Locus Sequence Typing (MLST) 398. Het 'niet typeerbaar zijn van deze stammen, bleek te berusten op de aanwezigheid van een nieuw methylatie enzym voor het DNA [1].

Om een inschatting te kunnen maken in hoeverre deze stam zich ook buiten de regio verspreid had en of ook andere dieren als varkens een risico vormden, zochten wij een groep mensen die verspreid door heel Nederland contact hadden met meerdere

soorten dieren. Dit resulteerde in een onderzoek naar MRSA dragerschap onder dierenartsen en studenten diergeneeskunde. De resultaten hiervan zijn weergegeven in **hoofdstuk 3**. Het bleek dat in 2005 het dragerschap onder dierenartsen en studenten diergeneeskunde hoger was dan onder patiënten bij opname in het ziekenhuis (4.7 versus 0.03%) [2]. Deze mate van dragerschap is vergelijkbaar met die van patiënten die overgeplaatst worden vanuit buitenlandse ziekenhuizen (respectievelijk 4.7% and 5%) [3]. Hieruit bleek dat vee-gerelateerde MRSA geen lokaal maar een nationaal fenomeen is.

De bevindingen van dit onderzoek werden bevestigd in de case controle studie van van Loo et al [4], die aantoonden dat ook contact met vleeskalveren een risicofactor voor MRSA dragerschap was. Deze bevindingen leidden tot een aanpassing in de landelijke richtlijnen van de Werkgroep Infectiepreventie (WIP). In juli 2006 werden patiënten die contact hadden met beroepsmatig gehouden varkens en vleeskalveren als risicogroep in de richtlijn opgenomen en dienden zij bij opname in het ziekenhuis te worden gescreend en geïsoleerd.

Om een indruk te krijgen of dit een Nederlands of internationaal probleem was, voerden wij een prevalentie onderzoek uit bij een congres over varkensgezondheidszorg in Denemarken (IPVS Kopenhagen). De resultaten hiervan zijn weergegeven in **hoofdstuk 4**. Deelnemers uit verschillende landen zoals Nederland, Duitsland, Spanje, Italië, België, Canada en Thailand bleken dragers te zijn van MRSA ST398, wat aantoont dat deze stam wijdverspreid is. Onder deelnemers was een duidelijk verband tussen het risico om MRSA drager te zijn en de tijd die in daadwerkelijk contact met de dieren wordt doorgebracht. Het gebruik van persoonsbeschermingsmiddelen zoals overjassen/overalls, handschoenen en maskers leek onvoldoende bescherming te bieden. Statistisch gezien was het dragen van een masker juist een risico om MRSA drager te zijn (odds ratio van 0.38; BI 0.12-0.99). Hoewel dit in eerste instantie onlogisch overkomt, kan het misschien verklaard worden uit het feit dat in stallen maskers vaak hergebruikt worden. Hun doel is voornamelijk om te beschermen tegen stofdeeltjes en niet tegen bacteriën. Bij hergebruik zou een gecontamineerd masker dan juist als bron van MRSA kunnen

dienen. Alle MRSA isolaten in deze studie en in de prevalentie studie in **hoofdstuk 3**, waren resistent voor tetracycline. 53% van de isolaten in dit onderzoek was zelfs resistent tegen 5 of meer groepen van antibiotica. Deze isolaten kunnen met recht multiresistent worden genoemd. Dit wijst erop dat het gebruik van antibiotica in de veeteelt een rol zou kunnen spelen bij de selectie van dit micro-organisme. Kijken we naar biologische boeren (**hoofdstuk 5**) die minder antibiotica mogen gebruiken, dan lijkt het dragerschap onder de veehouders lager te liggen dan in de reguliere varkenshouderij. Nader onderzoek heeft inmiddels aangetoond dat er inderdaad een verband is tussen het gebruik van antibiotica en het voorkomen van MRSA onder zowel veehouders als dieren [5].

De invloed van de aanpassingen in de MRSA richtlijn van de WIP op de gezondheidszorg

De wijzingen in de MRSA richtlijn van juli 2006 leidden onmiddellijk tot de vraag wat te doen met medewerkers in de zorg die contact hebben met vee, bijvoorbeeld omdat zij op een varkenshouderij wonen. Volgens de in 2005 (en nog steeds) vigerende richtlijn moeten medewerkers in de zorg zich melden als zij risico hebben gelopen om MRSA positief te worden, bijvoorbeeld door werk in een buitenlands ziekenhuis. Medewerkers worden dan gescreend op MRSA dragerschap en indien zij positief zijn, mogen zij niet in de directe patiëntenzorg werken en krijgen zij een dragerschapbehandeling. Om een inschatting te kunnen maken in hoeverre vee-gerelateerde MRSA nu werkelijk een probleem vormt onder medewerkers, voerden wij een steekproef uit onder werknemers in één academisch en vier perifere ziekenhuizen in het zuiden van Nederland, een gebied met veel varkenshouderijen (**hoofdstuk 6**). In deze ziekenhuizen had 4,4% van de werknemers uit de steekproef contact met varkens en/of vleeskalveren. In deze groep was het MRSA dragerschap met 1,3% laag (1 drager gevonden) en verschilde niet statistisch significant van dat in de controle groep (medewerkers zonder contact met dieren). Dit ondersteunt het besluit van de WIP om medewerkers in contact met vee niet op te nemen als aparte risicogroep in MRSA richtlijn. Incidenten kunnen echter

voorkomen en het is niet uitgesloten dat de eigenschappen van ST398-MRSA in de komende jaren gaan veranderen. In **hoofdstuk 7** beschrijven wij de eerste uitbraak met MRSA ST389 in een Nederlands ziekenhuis. In dit geval had geen van de MRSA positieve patiënten contact met vee maar was er wel een MRSA positieve medewerker die op het terrein van een varkenshouderij woonde, hoewel zij zelf geen direct contact met de dieren had.

MRSA ST398 dragerschap onder medewerkers wordt in de praktijk niet vaak aangetoond. Wanneer het wel gebeurt, is er sprake van een medisch-ethisch dilemma. Volgens de WIP richtlijnen mag een MRSA positieve medewerker niet in de directe patiëntenzorg werken, ongeacht de reden van de screening en ongeacht of de medewerker wel of niet voor verspreiding van de MRSA heeft gezorgd.

Het kan dus voorkomen dat een medewerker bij toeval MRSA positief wordt bevonden bijvoorbeeld wanneer hij of zij zelf als patiënt in het ziekenhuis belandt en aangeeft contact met vee te hebben. De medewerker mag dan niet meer werken totdat een succesvolle dragerschapbehandeling heeft plaatsgevonden. Bij veegerelateerde MRSA is de kans groot dat deze behandeling niet lukt, de medewerker wordt immers steeds opnieuw blootgesteld aan de bron. De vraag dient zich dan aan waarom deze medewerker, die bij toeval is gevonden en geen MRSA besmettingen heeft veroorzaakt, niet meer mag werken maar de collega op de afdeling die ook contact met vee heeft, gewoon door mag werken en niet gecontroleerd hoeft te worden. Dit wordt door veel mensen als een onrechtvaardig onderscheid gezien. Een actief screeningsbeleid onder medewerkers zou ertoe kunnen leiden dat gemotiveerd en bekwaam personeel aan de zijlijn komt te staan terwijl we deze mensen juist hard nodig hebben. Het verlies aan personeel zou de kwaliteit van zorg wel eens veel meer kunnen schaden dan de enkele vee-MRSA, mede omdat deze laatste zich toch wat minder gemakkelijk lijkt te verspreiden dan een aantal andere MRSA stammen [6]. Het is echter wel zaak deze situatie in de gaten te houden voor het geval dat overdracht tussen personeel en patiënt misschien vaker voorkomt dan tot nu toe gedacht. **Hoofdstuk 8** beschrijft de impact van MRSA ST398 op de aantallen MRSA dagers en MRSA infecties in het zuidoosten

van Nederland (regio Eindhoven). In deze regio steeg het aantal nieuwe MRSA positieve patiënten van gemiddeld 16 per jaar (juli 2002-juli 2006) naar 148 per jaar (juli 2006-december 2008). 82% van deze toename kwam voor rekening van MRSA ST398. Van de 80 gedocumenteerde MRSA infecties in 2007 en 2008, werden er 30 veroorzaakt door MRSA ST398 (38%). Van slechts 11 van deze patiënten was bekend dat zij contact met vee hadden. Dit geeft wel aan dat MRSA ST398 een groot beroep doet op infectiepreventie hulpmiddelen.

Het doel van het Nederlands search and destroy beleid is het voorkomen van zowel MRSA transmissie als MRSA gerelateerde ziekenhuis infecties. Het grootste deel van de kosten van dit beleid zitten in kweken, isolatiemaatregelen en de extra werklast voor de medewerkers aan het bed van de patiënt en voor de consulenten infectiepreventie. Vee gerelateerde MRSA ondermijnt het Nederlandse search and destroy beleid vooral in gebieden met veel varkens- en vleeskalverhouderijen. Ziekenhuizen in deze regio draaien op voor de extra kosten die in andere regio's van Nederland niet gemaakt hoeven te worden. De hierboven beschreven toename van nieuwe MRSA dragers met >900% is vermoedelijk een onderschatting van de daadwerkelijke toename van screeningen en isolaties. In tegenstelling tot het beleid bij patiënten uit buitenlandse ziekenhuizen, is routinematige dragerschapbehandeling van patiënten in contact met vee niet zinvol, zij worden immers steeds opnieuw aan de bron blootgesteld. Dit houdt ook in dat zij bij elk nieuw contact met de zorg weer als MRSA positief / verdacht moeten worden beschouwd en dus weer opnieuw gescreend en / of geïsoleerd moeten worden. De echte prijs van het beleid wordt in die zin misschien wel betaald door de patiënt. Isolatiemaatregelen kunnen namelijk leiden tot slechtere zorg [7].

De opkomst van de vee-gerelateerde MRSA kan niet los worden gezien van het gebruik van antibiotica in de veehouderij. Dit wordt geïllustreerd door het feit dat van de MRSA ST398 in deze observationele studie 70% resistent was voor meer dan 3 klassen antibiotica en 17% zelfs voor vier of meer klassen. Bij genetische analyse van MRSA ST398 door verschillende onderzoeksgroepen zijn veel verschillende resistentie genen aangetoond zoals *tet*(M), *tet*(K) en *tet*(L), die coderen voor

resistentie voor tetracyclines, *erm*A, B, C and T, verantwoordelijk voor resistentie voor clindamycine en erythromycin, *vg*A en *vg*C (lincosamide resistentie) *dfr*K (trimetoprim resistentie), aacA-aphD, aphA3, aadD en spc (aminoglycoside resistentie) en nog vele anderen [8-10]. Dit multiresistente phenotype staat in schril contrast tot dat van de community-associated MRSA stammen, die vaak alleen resistent zijn voor penicillines.

Zoals eerder aangegeven is het van belang om inzicht te hebben in eventuele nosocomiale transmissie van MRSA ST398. Hiervoor is een snelle en betrouwbare typeringsmethode nodig. PFGE met *Sma*I is niet mogelijk en hoewel typering met andere enzymen zoals *Xma*I wel kan, zijn deze technieken arbeidsintensief en kostbaar. *Spa*-typering is dat niet maar heeft onvoldoende onderscheidend vermogen in het geval van ST398 [11]. **Hoofdstuk 9** beschrijft de toepassing van Raman spectroscopie voor het typering van MRSA inclusief ST398. Ramanspectroscopie is gebaseerd op inelastische strooiing ofwel Raman-strooiing van monochromatisch licht. Door bacteriestammen te bestralen met een laser worden spectra gegenereerd die een vingerafdruk vormen van de moleculaire compositie van de stam. Door een historische collectie van 286 MRSA ST398 en 239 isolaten van andere PFGE clusters te analyseren, konden worden aangetoond dat Raman spectroscopie een onderscheidend vermogen heeft dat vergelijkbaar is met PFGE voor de bekende humane MRSA klonen. Er was een duidelijk onderscheid in spectra tussen MRSA ST398 en alle andere MRSA's. De MRSA ST398 uitbraakstammen (hoofdstuk 7) werden inderdaad als een cluster herkend en verder was er een opsplitsing binnen ST398 van 22 subtypes. Dit impliceert dat MRSA ST398 een zeer diverse moleculaire samenstelling heeft.

Dat MRSA ST398 verschilt van reguliere humane MRSA wordt bevestigd in **Hoofdstuk 10** waarin commerciële sneltesten op PCR basis niet in staat waren 20% van de geteste MRSA ST398 stammen te detecteren. Het ging dan voornamelijk om *spa*-type t567, een *spa*-type dat relatief vaak in de regio Eindhoven voorkomt.

Nadere genetische analyse van de stammen toonde aan dat er nieuwe en ongebruikelijke SCC*mec* types in deze collectie stammen voorkwamen, waarvan de herkomst nog moet worden opgehelderd. Sequentie analyse van deze elementen uitgevoerd door Li et al [12] toonde aan dat deze gevonden cassettes sterk afwijken van die van 'normale' humane klonen. Men kan beargumenteren dat dit erop wijst dat de overdracht van SCC*mec* cassettes in vee plaats vindt, mogelijk door of onder druk van antibiotica gebruik. In aanvulling hierop blijkt dat in de SCC*mec* cassettes van ST398 stammen ook genen zitten die geassocieerd zijn met de resistentie tegen zware metalen zoals koper en zink [12]. Omdat deze metalen in sommige landen aan het veevoer worden toegevoegd kunnen ze mogelijk – net zo als de antibiotica– aan de selectie van MRSA bij dieren bijdragen

Conclusies

MRSA ST398, het meest voorkomende clonaal complex in vee, is inmiddels gevonden in diverse diersoorten over diverse continenten. Overdracht is beschreven van andere dieren naar mensen en vice versa. Varkens, boeren en veeartsen in Canada, België, Italië, Duitsland, de Verenigde Staten en Denemarken zijn positief bevonden met dit type MRSA [13-18]. Gegevens over vleeskalveren laten identieke niveaus van besmetting zien [19]. MRSA ST398 heeft zich verspreid in een dierenziekenhuis in Utrecht, waarbij infecties zoals centrale lijn infecties en wondinfecties zijn beschreven bij paarden, net als bij ziekenhuis gerelateerde MRSA uitbraken bij mensen. [20] Naast de in dit proefschrift beschreven ziekenhuis infecties (hoofdstuk 7 en 8), wordt ST398-MRSA door Yu et al [21] genoemd als oorzaak van ziekenhuis infecties bij menselijke patiënten in China en wordt een dodelijke beademingspneumonie beschreven in Italië [22].

Toch lijkt MRSA ST398 zich nog niet heel gemakkelijk in de gemeenschap te verspreiden [23]. Hoewel isolaten met virulentiefactoren zoals PVL zijn beschreven [24,25] lijkt deze MRSA kloon nog het meeste op de ziekenhuis gerelateerde MRSA uit het verleden: niet heel virulent, maar wel in staat om infecties te veroorzaken bij

hen die er vatbaar voor zijn, door bijvoorbeeld onderliggende ziektes of wanneer de huidbarrière doorbroken is.

Het valt nog te bezien welke richting het opgaat met MRSA ST398 maar we moeten niet vergeten dat deze stam zich promiscue getoond heeft. Hij kan veel verschillende diersoorten koloniseren, hierbij ook infecties veroorzaken en is vaak multi-resistent. Helaas is er, i.t.t. tot wat sommige mensen beweren, geen makkelijke oplossing voor dit probleem. [26]. Het routinematige gebruik van antibiotica, bijvoorbeeld in biggen, zal zeker bijdragen tot selectie van deze MRSA in varkens. Er is gezamenlijk actie nodig van humane en veterinaire geneeskunde om verdere verspreiding van deze stam te voorkomen. In de tussentijd, is MRSA ST398 voornamelijk een arbeidsgerelateerd gezondheidsrisico voor hen die werken in de veeteelt, en dan in het bijzonder varkens en vleeskalveren.

De oude Grieken wisten dat er een verband was tussen infecties in mensen en dieren. Dat raakte lange tijd in de vergetelheid totdat het in de 19^{e} eeuw duidelijk werd dat micro organismen zoals *Mycobacterium tuberculosis* en *Bacillus anthracis* ziektes konden overbrengen van dier naar mens. Deze boodschap werd recent nog eens krachtig onderstreept door de grote Q koorts uitbraak in Nederland. Hoewel het aantal gevallen van mensen met MRSA ST398 infectie daarbij in het niet valt, moet de opkomst van deze stam in de context van een groter probleem worden gezien, namelijk dat van de antibiotica resistentie. Voorbeelden daarvan zijn de vancomycine resistente enterokokken (VRE), multiresistente *Salmonellae* en recent de ESBL positieve, Shiga toxine producerende *E. coli* (STEC / EHEC). Al deze gevallen onderstrepen een duidelijke boodschap: antibiotica moeten spaarzaam gebruikt worden, namelijk voor het behandelen van bacteriële infecties. Antibiotica zijn te kostbaar om gebruikt te worden als goedkope oplossing. Anders bestaat het risico dat we binnen enkele tientallen jaren terugkeren naar de pre-antibiotisch tijdperk.

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Tot slot wil ik toch even de kinderen noemen, ja, die met 4 pootjes en een vachtje. Zij vonden het prima als ik thuis zat te klussen, gingen graag mee als ik even uit moest waaien en waren gewillig slachtoffer als ik even moest knuffelen. Misschien wel hun belangrijkste boodschap: geniet van het moment!

Curriculum Vitae Mireille Wilhelmina Huberta Wulf

Mireille Wulf werd geboren 24 maart 1970 te Eindhoven. Na het VWO-gymnasium in Deurne, studeerde zij Medische Biologie aan de Universiteit van Utrecht. Daar maakte zij voor het eerst kennis met de microbiologie tijdens een onderzoeksstage over virulentiefactoren bij pneumokokken. Omdat het vak van arts-microbioloog haar wel wat leek, besloot zij na haar doctoraal Medische Biologie ook Geneeskunde te gaan doen. Na haar studie werkte zij 2 jaar als internist in opleiding maar het laboratorium lonkte en per 01-01-2001 kon zij starten zij met opleiding tot arts-microbioloog in het UMC St. Radboud. Na het afronden van haar opleiding werkte zij daar nog anderhalf jaar als arts-microbioloog met infectiepreventie in haar portefeuille onder de bezielende leiding van Prof. Dr. Voss. Van 2007 tot 2011 werkte zij bij de Stichting PAMM, laboratorium voor Medische Microbiologie en Pathologie in Veldhoven en was zij tevens staflid van het Catharina Ziekenhuis te Eindhoven met als aandachtsgebied de infectiepreventie. Per 1 november 2011 werkt zijn dichter bij huis in het Viecuri Medisch Centrum te Venlo. De interesse voor de vee-gerelateerde MRSA werd gewekt in Nijmegen en de verhuizing naar het epicentrum van de varkenshouderij in Nederland stimuleerde deze interesse verder, wat resulteerde in dit promotieonderzoek. Mireille woont met haar partner Hans Schipper en hun drie Siberische huskies in het kleine, maar fraaie Maasdorp Beesel.

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