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SPREAD OF METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA) IN DAIRY COWS IN ASSOCIATION WITH PIG FARMING

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ABSTRACT

Among bacteria *Staphylococcus aureus* plays a very relevant role both in humans and animals infections. It is present primary on the skin and through direct contact it can be transmitted to new hosts and can also be spread wherever in the environment. As a consequence, *S. aureus* reservoir are living carriers and contaminated inanimate objects too (e.g. needle sharing among drug users, used disposable materials in health care facilities, foreign bodies in skin wounds and even dust in contaminated environments). *S. aureus* causes a variety of infections generating abscesses in many body districts, in general it is the most frequent responsible for skin and soft tissue infections (SSTIs). Pneumonia, fasciitis, cellulitis, empiema of natural cavities (e.g. gallbladder), osteomyelitis, arthritis, implanted-device associated infections are only some of the most common diseases caused by *S. aureus*. Extremely severe and life-threatening are endocarditis and blood stream infections up to septicemia. *S. aureus* can be acquired everywhere but is a typical heath care-associated infection. Analogies can be found with *S. aureus* infections regarding animals, especially companion animals whose hospitalization is becoming more and more similar to that reserved to humans. It is present in a wide range of animal species, including dogs, cats, rabbits, horses, cattle, pigs, poultry, and exotic species, both as a cause of infection and in healthy carriers. Identification of MRSA in various species and in food has led to concerns about the roles of animals, both pets and livestock, in the epidemiology of MRSA infection and colonization in humans.

A special issue regards dairy production animals, in particular cows, for which *S. aureus* is the most important udder pathogen. The present work means to investigate the presence, importance and origin of *S. aureus* affecting dairy cows belonging to strains defined methicillin-resistant (MRSA). Since pigs has been found the first food-producing animal species carrying MRSA and recognized as a reservoir, the likely relationships with dairy herds present on the same area will be discussed.

INTRODUCTION

Staphylococcus (*S.*) *aureus* causes a wide range of severe and economically-important diseases in human and veterinary medicine (Leonard and Markey, 2008; Safdar and Bradley, 2008). The bacterium is a colonizer of the skin and mucosae from which it can invade multiple organs. In humans, *S. aureus* is a persistent resident of the human nose in 20% of the healthy population, and intermittently carried by another 60%. It is a common cause of community-acquired skin infections, and a major cause of hospital-acquired infections such as surgical and catheter-site infections, bacteremia and pneumonia (Sung et al., 2008).
In humans, prevalence of *S. aureus* infection varies widely between European Member States, among hospitals and inside hospital. The reasons for the difference are likely due to the level of screening, isolation and monitoring of patients and staff in hospitals, with the Dutch having the most pro-active system over the last decades. There is a shortage of quality data investigating infection and/or carriage rates in the community, but occurrence appears to vary substantially with geography (EFSA, 2009).

In livestock *Staphylococcus aureus* is an important cause of mastitis, skin and soft tissue infections (SSTI) and to lesser extent infections of the locomotory system. Surgical site infections (SSI) in which *S. aureus* is isolated have been increasingly reported in small companion animals and horses.

Methicillin was introduced in 1959 to treat infections caused by penicillin-resistant *S. aureus*. Soon after resistance phenomena arose. MRSA has been firstly described in the UK in 1961 as a human pathogen, strictly confined to health care unit and hospitalized patients (HA-MRSA). The strain was typed as the Sequence Type (ST) 250. Since then MRSA importance rapidly and steadly increased in hospital settings, new ST emerged and new typing methods became necessary to manage the outbreaks. This is a challenge still urgent.

A Methicillin-Resistant *Staphylococcus aureus* infection is essentially the same as a *S. aureus* one, but for antimicrobial resistance. MRSA originate from a methicillin-susceptible *S. aureus* (MSSA) that acquired a mobile genetic element called Staphylococcal Chromosome Cassette (SCC*mec*). SCC*mec* carries the *mec*A gene coding for a Penicillin Binding Protein 2 (PBP-2) with reduced affinity for beta-lactams which is the reason of resistance. The ancestral origin of *mec*A gene has long been searched. It is possible that *S. fleurettii*, belonging to *S. sciuri* group, could be the origin and animal staphylococci a reservoir. *S. aureus* could has been a secondary acceptor of SCC*mec* coming from CNS. This mobile genetic element consists of the *mec*A gene

and a functional *ccr*-complex coding for enzymes (recombinase, invertase etc.) responsible for the release from a staphylococcal genome and the insertion into another. The third part is the variable J-region with no essential genes but that can collect series of genes conferring resistance to other antimicrobial classes (Fig. 1). The J-region defines the type of SCC*mec* and characterizes the specific strain. To date, eleven SCC*mec* can be distinguished on the basis of the different key genetic element present: the complex of *mec*A and its regulatory genes.

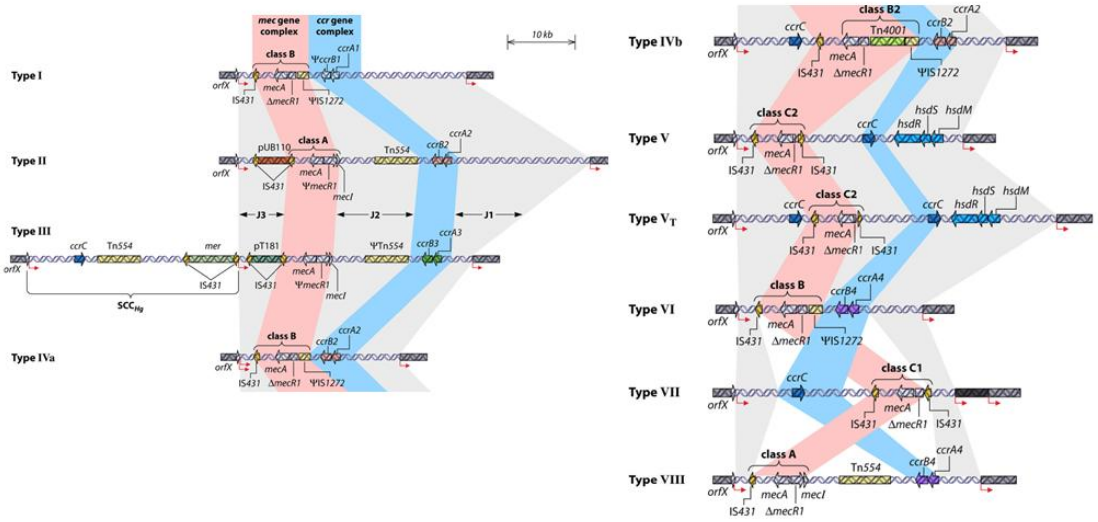

Figure1 Classification scheme of some of the SCC*mec* type, adapted from David & Daum (2010). The pink zone is the *mec*A gene, in blues there's the *ccr* gene complex and in grey the J-region.

In general the presence of SCC*mec* can be an advantage if drugs are used, but also a limit in absence of treatment. The strains carrying this genetic element have a relatively slower growth rate and so they may be less fit to survive in a competitive environment (Monecke et al., 2011). For instance, SCC*mec* IV is very common in a wide variety of MRSA lineages and other staphylococci (i.e. *S. epidermidis*) and this could be due to the ability to be easily transmitted from a strain to another and to represent a low cost in terms of fitness. The evolution of this mobile genetic element is in progress and apparently it evolves independently of their bacterial host genome, depending on different selective forces. The types recognized are by now eleven (Shore et al., 2011). SCC*mec* confers an advantage to the microorganism it resides in, both thanks to resistance to antibiotics and to the additional virulence factors it carries, in accordance with the genes acquired and inserted.

Also Panton-Valentine Leukocidin coding genes undergo a host-independent evolution. They resides on phages and so they can be found in a huge variety of clonal complexes (CC).

Staphylokinases are of particular strategic importance and improves microbial fitness in pathologic condition conferring the ability to penetrate deeper host tissue. For this reason PVL-positive MRSA are specially involved in STTI (Skin and Soft Tissue Infections), less frequently in necrotizing pneumonia. However in setting where such strains are particularly diffused it in possible to find them involved in bloodstream infections.

The National Nosocomial Infections Surveillance System (NNISS) in the US reported that in 2003 over 60% of *S. aureus* isolated in Intensive Care Units (ICUs) were MRSA. In Europe is reported a MRSA prevalence in bloodstream infections ranging from the very low 1% in Northern Europe countries to 50% in Mediterranean countries (Kock et al., 2010). The US Center for Disease Control (CDC) reported in early '80s MRSA as a problem limited to large urban hospitals with a prevalence of 5-10%. By the 1990s, MRSA prevalence rates among both large and small hospitals had increased to 40% and 20%, respectively. Monitoring pathogens occurrence in STTI patients in hospital worldwide in a 7 years period revealed that *S. aureus* is the most frequent and that MRSA increased in North and South America, gaining 50 and 40% respectively, while remained steady around 20% in Europe. Otherwise, in the latter continent, prevalence in extremely variable among Member States, ranging from 0.8 to 50%. Data suggest that MRSA is becoming a more and more important pathogen, with a continuously increasing prevalence and alarming spreading among outpatient, besides inpatients (Bassetti et al., 2009).

Since 1990s new strains, joined under the definition of Community-Associated MRSA, emerged specially in the U.S. They were responsible for a notable increase of infections incidence among general population, that is people without evidence of exposure to health care environment. The two groups of strains can be distinguished on a molecular base and of particular interest is the size of the staphylococcal chromosome cassette (SCC*mec*) which is large in type I, II and III for HA-MRSA and smaller in CA-MRSA (types IV and V). CA-MRSA seems to have and elicited mobility of SCC*mec* and frequently carries Panton-Valentine Leukocidin (PVL) genes. The average age of infected people is different too: HA-MRSA involve usually eldest patients whereas CA-MRSA infect young people and account to 75% of *Staph. aureus* infections in children in the USA. These make communities a large reservoir for new infection so that any efforts to control hospital circulation could have no success without interventions on spread at the community level.

A summary of differences between HA-MRSA and CA-MRSA is proposed in Table 1. Substantially CA-MRSA, unlike HA-MRSA, is remarkably fit and able to spread within

communities; it is virulent and often susceptible to multiple narrow-spectrum antimicrobial agents. It occurs in patients without classical risk factors. Outbreaks have been reported among members of cultural minorities, persons who have close contact, especially if there is skin trauma (e.g. athletes), and people living in closed communities (e.g. maternity wards, military establishments, day-care facilities and prisons). MRSA is normally transmitted from person to person by hand or skin contact in general, sharing contaminated items (e.g. razors, athletic equipment, soap or towels) or via contaminated surfaces David and Daum (2010). While HA-MRSA is multi-drug resistant, CA-MRSA is usually susceptible to fluoroquinolones, clindamycin, trimethoprim–sulfamethoxazole and tetracycline. Apart from molecular characterization, in hospital setting a MRSA is defined CA when it is isolated at least 48 hours after admission to the hospital (Bassetti et al., 2009). CA-MRSA is usually reported more virulent than HA-MRSA in terms of morbidity and mortality. It appears as skin lesions but also necrotizing pneumonia might be a severe consequence of such infection. A higher growth rate compared to HA-MRSA constitutes an advantage for competition and even substitution in clinical settings when CA-MRSA carriers are admitted to hospital. In the US with the spread of clone USA300 the diffusion of CA-MRSA took the size of an epidemic. Whereas in Europe the most common clone in ST80 and with a lower prevalence.

Table 1: a comparison between HA-MRSA and CA-MRSA based on some main features. Adapted from Bassetti et al., 2009 and David and Daum (2010).

	HA-MRSA	**CA-MRSA**
Healthcare onset of infections	Yes	no
Mean age of infections	Older	younger
Prevalence of SSTIs among other infections	35%	75%
Staphylococcal Chromosomal Cassette(SCCmec)	Types I, II, III	Types IV, V
	Large, reduced mobility	Small, more mobile
Resistance towards other drug classes	Many agents	Some agents
Panton-Valentine Leukocidin (PVL)	Rather absent (5%)	Typically associated (~100%)

WHY IS MRSA SO DANGEROUS AND FEARED?

Since its emergence, MRSA had (and continues to have) a tremendous impact on morbidity and mortality in hospitals. In a survey at teaching hospital Policlinico Umberto I (Rome) on patients admitted at Intensive Care Unit, MRSA resulted the second most common cause of blood stream infections (14.9%), preceded by CNS (26.2%) and followed by *Pseudomonas aeruginosa* (13.5%) (Orsi et al., 2012). Among a bacterial population of 128 *S. aureus* isolated in patients in four hospitals in Italy, 11out of 52 patients with bacteremia were infected by MRSA (21%), while 21 out of 76 patients (28%) with diagnosis of infective endocarditis by *S. aureus*, were infected by MRSA (Campanile et al., 2012).

As with other multi-resistant infections, MRSA infections are associated with high costs and extended hospital stay. Patients with surgical site infections due to MRSA had a greater mortality rate than patients infected with MSSA (odds ratio (OR) 3.4, 95% CI 1.5–7.2). Patients infected with MRSA had a greater duration of hospitalization after infection ($p < 0.001$). Median hospital charges were US$ 29,455 for control subjects, US$ 52,791 for patients with MSSA SSTI, and US$ 92,363 for patients with MRSA SSTI ($p < 0.001$). Patients with MRSA SSTI had a 1.19-fold increase in hospital charges ($p = 0.03$) and had mean attributable excess charges of US$ 13,901 per SSTI compared with patients who had MSSA SSTIs (Engemann et al., 2003). In another study, patients with MRSA infections had a trend toward longer hospital length of stay (15.5 vs. 11 days; $p = 0.05$) and longer antibiotic-related hospital length of stay (10 vs. 7 days; $p = 0.003$) (Kopp et al., 2004). There are clear evidences for the expensive effect of MRSA on health care management.

An inadequate antimicrobial therapy and the time of initiation of a correct one have a strong impact on prognosis and mortality rate in patients. A rapid diagnosis and availability of the susceptibility profile should allow to improve prognosis and reduce mortality. A bacteriological standard diagnosis, in blood culture for instance, requires at least two days and is affected by early antibiotic therapy. The use of PCR for rapid detection of *mec*A gene in blood and other matrices improve sensibility, specificity and reduce time for effective response. Nevertheless, molecular biology technique application is another increasing cost in MRSA cure.

Moreover, the estimated cost for treating a hospitalized patient infected by MRSA ranges from 6,000 to 10,000 € (Diller at al., 2008), considering the arguments above proposed (longer hospital stay, confinement of infected patient, prolonged antimicrobial treatment with expensive drugs). The so-called search-and-destroy policy, which consists in early identification and cure of infected subject, appears convenient and even cheaper as Gavaldà et al. (2006) evaluated that

the cost of MRSA admission screening in an entire hospital in Spain is equivalent to treating four MRSA patients. Screening at hospital admission is particularly essential considering that by now most MRSA strains come from the community and is increasing the role of strains from livestock, as will be discussed in this work.
This, there are clear evidences of the importance of prevention at all levels too.

MRSA AND ANIMALS

Phenotypic (phage typing, biotyping etc.) early and molecular biology techniques later have demonstrated that among *S. aureus* there are some strains host-specific and some others (the minority) shared among different hosts. Usually in host-jump the lineages face a rearrangement of their virulence factors, switching towards an assessment more fit to the new host. ST5 in an example of lineage which is recently shifted from humans to poultry. While most strains of *S. aureus* show a considerable level of host-association, the most common LA-MRSA strain (ST398) fails to do so (Holmes and Zadoks, 2011).

The role of animals has not been fully characterized. ST398 has been recognized originated from animals (van Loo et al., 2007) even if there are molecular evidences that the lineage was human at origins and probably passed to animals following a partially independent evolution, under the pressure of blanket drug treatment (Price et al., 2012). Such MRSA strain was and keeps on to be part of the natural nasal flora of pigs. It has been suggested that MRSA could originate from humans and animals are at risk if in contact with them. This seems to be true for pets in which MRSA strains are those isolated in humans. While in production animals the strains are distinct from the human ones, even if with some exceptions that witnesses an epidemiology not so easy to trace (Türkyılmaz et al., 2010). The relationships between humans and animals as target or reservoir of MRSA must be seen in the view of zoonoses and in the concept of "One Health" policy. In other words, human and animal infections have not to be considered disjointed one the other. Evaluation of risk and typing of the strains involved should be the tool for understanding and preventing uncontrolled spread of hard to treat bacteria.

Since 2003, the emergence of non-typable MRSA (NTMRSA) in pigs, also called Livestock Associated MRSA (LA-MRSA), focused attention on the risk that MRSA could derive from contact with livestock and food animal consumption (Lee, 2003). Typability or not typability by Pulsed Field Gel Electrophoresis (PFGE) with *Sma*I as the enzyme for genome digestion

represented a marker for LA-MRSA and the first line way to distinguish it from HA-MRSA and CA-MRSA. Also the analysis of geographic distribution of cases and controls compared to pigs density sounds as a conformation: on a map NTMRSA and typable MRSA isolates correlates with the density of the pig and human populations, respectively (van Loo et al., 2007). Case-patients more often lived in rural areas and indicated more frequent contact with pigs or cattle than did controls. Controls were more often associated with healthcare facilities. Contact with pigs and cattle was demonstrated a significant statistical variable, with a calculated odds ratio (OD) of 9.4 and 13.5, respectively (van Loo et al., 2007). Authors dealing so deeply with these issues referred to The Netherlands or Denmark, but they also assumed that the problem is not limited to those countries. Moreover, travelling, livestock trading and epidemiological situations comparable to those described could yield similar effects. It is so clear that close relation with farming and even contact with livestock represents a risk for humans. MRSA (any MRSA type) carriage or mere transient contamination predispose to infections, as explained above. The infections eventually established can be severe (Soavi et al., 2010; Lozano et al., 2011) and prevention is necessary. The presence of ST398 in human patients has been demonstrated but the extent of spread among the community is partly unknown. Determination of prevalence in animals and humans at local and international level is necessary in order to formulate adequate preventive measures and recommendations.

In the following chapter an overview will be attempted on available literature about pet and production animals MRSA diffusion and infection prevalence, with special regard to dairy cows and pigs and in particular from a zoonotic point of view.

MRSA IN COMPANION ANIMALS

Up to late 1990s MRSA had been only a sporadic relief in clinical infections of companion animals. In recent studies the colonization of dogs and cats, considering the general population, remain low ranging from 0 to 2,1 %. When animals in particular difficult situations (rescue kennels, veterinary hospitals etc.) are taken in consideration the prevalence rises to 9 % (Loeffler et al., 2011). MRSA is usually associated to skin infections, especially after surgery, and so *S. intermedius, S. pseudointermedius* and *S. schleiferi*. Surgery of fracture and soft tissue are also followed by complication due to MRSA contamination coming from the skin. Contamination of nose and hands is a risk factors for more severe MRSA infection in a human hospital as well as the carriage of MRSA on animals coat predisposes to infections in case of skin poor conditions and bad preparation to surgery.

Transmission of bacterial strains between companion animals and their owners has been demonstrated in several instances. Molecular analyses have shown the presence of indistinguishable MRSA strains in pets and humans living in the same household. An important issue to be underlined at this regards is that both humans and animals are more often colonized than infected by MRSA. Such a colonization can be transient or lasting for different time depending on several aspects like exposure intensity or frequency. Determining the level of colonization in different and specific population is useful to know MRSA burden and the risk of transmission. For instance, Faires and colleague (2009) reported a significantly different MRSA prevalence between general population (1-2 %) and pets owners (18 %). Pets can be colonized by any type of MRSA. The epidemiological survey about outbreaks is a precious source of evidences on MRSA way of diffusion. It should include molecular analysis of the respective strains and the critic collection of information about persons and animals involved. The following two cases are interesting example (Nienhoff et al., 2009).

Case 1. A six-months-old female dog without sign of infection was found positive for MRSA carriage at nose-throat level. The survey to discover the source of colonization involved the owner (a veterinarian working in pigs field) and other three member of the family. The strain typing on dog and the owner isolates revealed a cross-contamination with the same MRSA, ST398 (typical LA-MRSA, at first isolated in pigs) of the *spa* type t034. As a peculiar feature on ST398, both strains was digested by *Apa*I but not *Sma*I enzyme in typing by PFGE.

Case 2. An eleven-years-old male dog with no signs of infection too was found positive for MRSA carriage at nose-throat level. The dog was daily in contact with nurses that provided care for a foot ulcer to an elderly woman living in the same household. The strains isolated from dog throat and eye ulcer and from woman wound were identical and belonged to ST225, the second most diffused MRSA type in German general population, and to t014, typeable both by *Apa*I and *Sma*I digestion.

These two cases demonstrate the ease of cross-contamination among humans and animals. The direction of transmission can be suggested by isolated types, for instance in case 1 the path possibly followed by ST398 should have been from pigs to farmers and the veterinarian and from the later to the household members, included the dog. Whereas in case 2 the source of infection is the elderly woman or probably the nursing personnel in daily contact with critical patients. Dogs and cats can also be the source of infection for humans through friendly contact, animal bites and in health facilities through contact pet-veterinary staff (Loeffler et al., 2011). A healthy cat harboring MRSA on the skin has been the first reported case of zoonotic transmission from companion animal to human vulnerable patients (Scott et al., 1988). However, the companion animal strains differ from those of production animal and coincide with those effectively present in their owner (in case 1 the dog was infected by a LA-MRSA because its owner had been exposed to livestock due to his job). This is because pets care implies a far closer contact between owners and animals than in animal husbandry and so MRSA acquisition is more likely a "humanosis" than a zoonosis (Morgan, 2008).

Horses, differently from other pets, are colonized or infected by MRSA types quite different from the lineages predominant in humans in the corresponding areas (Loeffler and Lloyd, 2010). Moreover the link between horses and humans seems to deserve a chapter apart.

MRSA IN HORSES

The first description of MRSA in horses reported sporadic infections intended to be unusual events involving direct transmission from humans. As with pets, further investigation revealed that colonization of a certain percentage of healthy horses is a common fact. Most colonized horses do not develop clinical infections, but they are at risk at the time of hospital admission for the establishment of a wide variety of infections, some of them already listed for human patients (pneumonia, metritis, omphalophlebitis, sinusitis, bloodstream infection, invasive device

infection, osteomyelitis, tenosynovitis, metritis, and mastitis). MRSA-colonized horses are the potential source for transmission to other horses, humans, and other animal species. The epidemiological and biological specificities of each animal species reflect on the strains that tend to be involved. Most initial reports of MRSA in horses involved ST8 and other strains that had been epidemic among humans but currently not so common. Their remarkable presence in horses can be an expression of their adaptation to this species and to their major fitness to persist in horses than in humans (Weese, 2010). Horse MRSA commonly belong to ST8 and related STs within clonal complex (CC) 8. A typical horse clone is USA500 (Morgan, 2008), that emerged as an HA-MRSA clone in the USA but is now infrequently recovered in human patients. More recently, studies from Europe and Canada reported horses to be colonized by LA-MRSA ST398 (Cuny et al., 2008; Tokateloff et al., 2009). Similarly to dogs and cats, the prevalence among healthy animals in normal population is significantly lower than that of animal in teaching hospital (16 %), which are probably sick, at least compromised and in contact with other animals with possible exchange of pathogen (Baptiste et al., 2005). Reports indicate that antimicrobial exposure is a risk factor both for being colonized with MRSA on admission to an equine hospital and for becoming colonized during hospitalization (Weese, 2010). This is an issue that matches with the reliefs in humans nosocomial infections.

MRSA AND DAIRY COWS

S. aureus is one of the major udder pathogens in dairy cows, responsible for intramammary infections (IMIs) destined to persist in a subclinical way for long time. Chronic subclinical infection can know from mild to high increase of Somatic Cell Count (SCC) until flare-ups in clinical mastitis. In general, damage to secretive tissue is relevant since *S. aureus* infections are deep-seated in the parenchyma and production losses weigh on a farm economy due to discarded milk and in any case to reduced milk quality and quantity. Epidemiology talks about *S. aureus* as a contagious pathogen for which the main risk of diffusion is the presence on infected quarters, for the other quarters, and infected cows for their herdmates. The crucial moment for contagion is daily milking, the hazardous place in milking parlor and the vectors are procedure and personnel that prepare udder teats to the work of milking machine. Finally, the efficiency of the latter is another ring of the chain that should protect bovine udders from *S. aureus* and that cannot be renounced. Basing on these cornerstones, in 1960’s the 5-points program and later the

extended 10-points program in 2001 developed measures to reduce udder infections. For contagious organisms such as *S. aureus*, proper milking procedures, use of postmilking teat disinfectants, use of gloves by the milkmen, biosecurity to prevent introduction of pathogens, and segregation or culling of chronically infected animals are important aspects of these plans. Failure to control *S. aureus* mastitis may be caused by failure to implement the 10-point program correctly or completely (Barkema et al., 2006). Usually lack of all infected animals detection through bacteriological culture and poor response to antimicrobial treatment are also proposed to justify failures.

Contagiousness of *S. aureus* seems to be disputable to some extent. Molecular studies have actually demonstrated the presence of several strains within a herd and not only a main dominant strain transmitted cow-to-cow. So, presence of multiple strains should indicate that there are extramammary sites where *S. aureus* can originate (Capurro et al., 2010). If the presence of infected animals is the main source of new infections, the ubiquity of this pathogen opens the discussion of environment, in the wider sense meant (cows' skin, farm environment, bedding materials, insects, air, other animals) as a possible reservoir of *S. aureus* fit to colonize udders.

There's no evidence that a Methicillin- resistant *S. aureus* may has a different epidemiological behavior (Vanderhaeghen et al., 2010). In the present work a wide panel of samples has been analyzed to search MRSA: quarter and bulk milk samples, milkers and personnel in contact with pigs and cows, cows and pigs nostrils, environmental dust in the air and on facilities. The aim, according to what written above, was to consider all possible source of infection both for contagious and environmental route of contamination. The only novel implication of MRSA compared no MSSA is a risk from a public health point of view. The only novel complication is an additional therapeutic limit since MRSA displays multiple resistance, first of all towards beta-lactams, which are a drug class heavily used in mastitis approach.

It is possible that some virulence factors involved in udder tissues aggression could be associated to the presence of the SCCmec, the mobile element carrying mecA gene and other genes, variable in size and number. (Monecke et al., 2011) Those genes, as introduced in the specific chapter, can be involved in antimicrobial resistance and pathogenicity as well.

The first description of a Methicillin resistance in *S. aureus* in bovine mastitis came from Belgium in 1972 on the basis of antimicrobial sensibility. Later biotyping of the strain recognized it to be of human origin. In Switzerland MRSA was found in 1.4% of mastitis cases and belonged to ST1 (Huber et al., 2010). In Japan only four strains belonging to CC5 were

found among 363 *S. aureus.* An unexpected prevalence of 10% among 118 isolates was found in Belgium among farms surveyed for mastitis cases. A herd-level, farms where MRSA was found the prevalence ranged from 3.9 to 7.4%. All the isolates were ST398, divided in *spa* types t011 (the most prevalent in Belgian pigs, see Figure 2) and t567. In this sense, apart from prevalence the survey on mastitis isolates types seems to reflect the regional epidemiology of MRSA in pigs.

In Turkey 16 out of 93 isolates from bovine mastitis were MRSA. All were typeable by PFGE and digestion with *Sma*I and none harbored PVL genes. The isolates didn't belong to LA-MRSA typical lineage ST398 and MLST confirmed instead ST239 and ST8 (Türkyılmaz et al., 2010). Such a high proportion of positive strains accompanied by high level of drug resistance is partially justified by the author with the assumption that their laboratory constitutes the extreme option for farmers and veterinarians as they receive samples only after treatment failure.

Whereas bovine mastitis is primarily associated with a limited number of CCs of *S. aureus*, many MRSA strains from cattle (CC1, CC5, CC8) did not belong to bovine-adapted CCs (Holmes and Zadoks, 2011). An example is ST1 t127, belonging to the typically human CC1, which has been found in the nose of a bull (Huber et al., 2010) and in a case of bovine mastitis (Pilla et al., 2012). These observations suggest that this strain may possess characteristics that enable it to cross the host species barrier more readily than other lineages. Also ST398 found in Belgian and German studies (Vanderhaeghen et al, 2010; Feßler et al., 2010) has been recently recognized as an ancestral human MSSA lineage, that then became MRSA acquiring a SCC*mec* element (Price et al., 2012). Thus, the risk for dairy cows could be represented both by transfer of MRSA strains from other animals and humans, and by transfer of the mobile genetic element conferring resistance from *S. aureus* or other staphylococci. To date, no examples of transfer of SCCmec between bovine Methicillin-Resistant Coagulase-Negative staphylococci (MRCNS) and *S. aureus* have been documented, which may indicate that the risk is low or that relevant studies have not been conducted. Nevertheless, CNS are very common isolates in bovine milk even though without clinical importance. Moreover, various species of MRCNS are present in pigs farms (Vanderhaeghen et al., 2012) with the risk of cross-contamination in case of concomitance of pigs and dairy cows.

MRSA IN PIGS

Swine is the main studied livestock species in the regards of MRSA. It has been firstly identified as a pig concern in the Netherlands when a young girl was found carrying MRSA as a result of a preventive screening before thoracic surgery. Her family members were positive too and the fact that they lived in a farm raising pigs opened the question about the zoonotic link between humans and animals (Voss et al., 2005). A wider survey was engaged involving farmers and pigs. During a professional meeting 23% of farmers were MRSA positive. The difference between prevalence in regional pig farmers and the general Dutch population was great: >760 times. All the isolated strains (from patients, their family and from pigs) shared the feature to be not digested by the enzyme *Sma*I typing them through PFGE.

This first study demonstrated at the same time that raising pigs could be a risk factor to be colonized by MRSA and that circulation of the same strain among humans is possible. The identification of the new non-*Sma*I typeable strain would in brief lead to the definition of the Sequence Type 398 as Livestock Associated MRSA (LA-MRSA).

ST398 is now the most diffused and typical MRSA among production animals. Guardabassi in 2007 announced that the detection of ST398 in Danish pigs suggests that this new emerging zoonotic bacterium is rapidly spreading in the pig population in Europe, according to the importance of Danish pigs exportation all over Europe. Some other clones has been found associated to pig farming (ST1, ST9, ST97) but at lower frequency (EFSA, 2010). ST1 in particular is a common human clone, found in pigs at the slaughterhouse as well, which could be an example of human to pig contamination or otherwise could be an ancient human clone recently adapted to animal hosts (Battisti et al., 2010). A survey promoted by the European Food Safety Agency (EFSA, 2010), together with the control of Salmonella, used environmental dust as an indicator of contamination of pig facilities. The risk of contamination was found significantly associated with pig holding size and the country in which the holding is seated. A breeding holding and a production holding housing respectively more than 400 and 100 breeding pigs were twice as likely to be MRSA-contaminated compared to breeding and production holdings with less than 100 breeding pigs. Biologically plausible reasons for a positive association are that larger holdings with breeding pigs probably need important genetic input resulting in more trading contacts and purchases of replacement breeding stock, which may put them at a higher risk of introduction of agents of either animal or public health significance.

Similarly, within-herd circulation of pathogens is more likely in larger holdings, as more potential shedders of the pathogen are present. About the country-factor, in the statistical models applied two-thirds of the total variance in MRSA prevalence must be attributed to the country. Actually, the survey showed that there were countries with a high prevalence as well as countries with low numbers or not at all MRSA-positive holdings (EFSA, 2009).

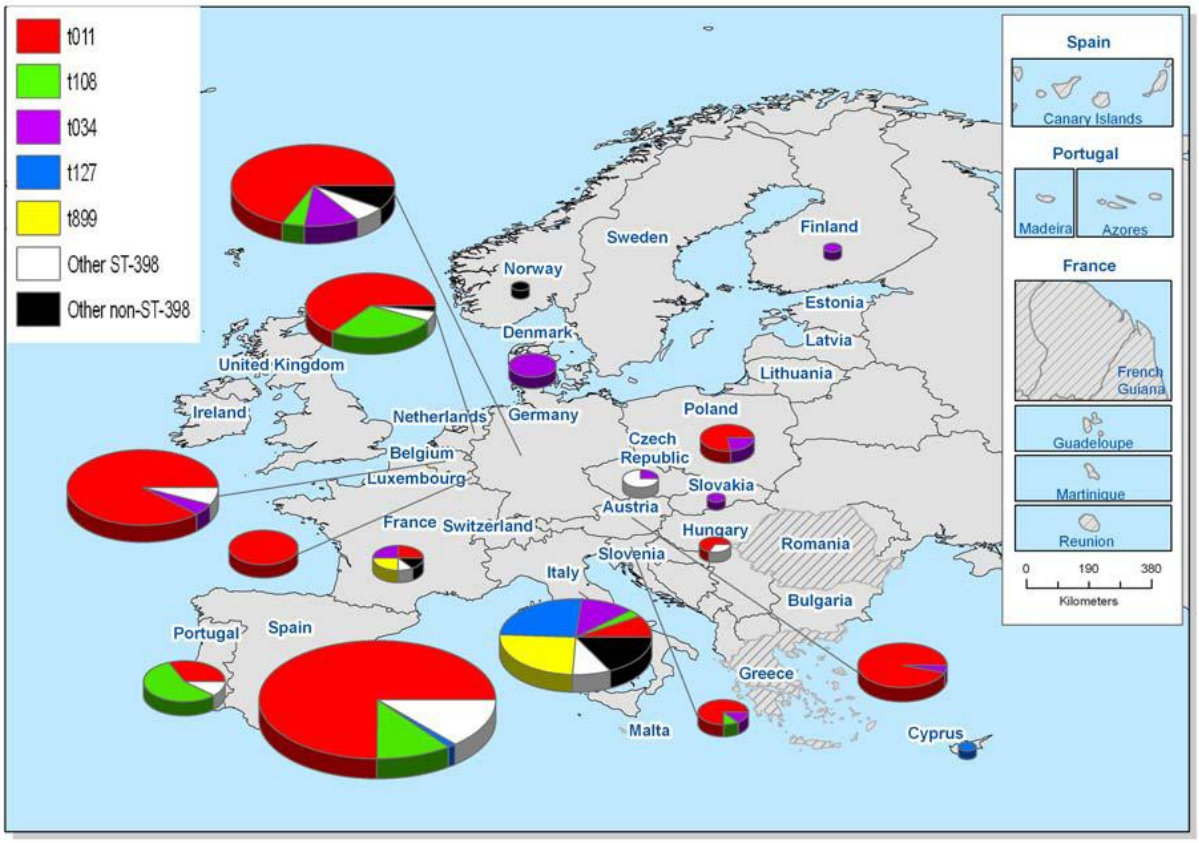

Figure 2: The distribution of MRSA *spa*-types in environmental dust samples collected from breeding and production holdings in the EU (from EFSA, 2010).

Other factors were found associated with MRSA prevalence: higher density of pig farming in the area, the entity of exportation/importation of living pigs in the regarding the member state, the type of holding and substitution policy of sows. The survey offered an overview on the geographic distribution and specificity of the *spa* types revealed (Figure 2), that will be useful to compare MRSA found in this thesis.

The typical staphylococcal pathogen for pigs is *S. hycus* whereas *S. aureus* has not in general a significant role for pig health status and rarely has been reported as a cause of clinical infection in pigs. MRSA isn't an exception. MRSA in The Netherlands was found to be the causative

organism during an outbreak of exudative epidermitis, normally due to *Staphylococcus hyicus.* All isolates were *spa* type t011, ST398. *S. hyicus* exudative epidermitis disease has a mortality of 20%, but the MRSA-infected piglets were successfully treated with enrofloxacin. Apart from this report, the real threat of MRSA presence is the contamination of food products and of the environment that becomes a source of dissemination for working people and their families, all along the production chain. A Dutch survey on pig production chain (Broens et al., 2010) conducted on 48 herds belonging both to complete and incomplete chain trading live animals, revealed the presence of MRSA in all types of farm and a total prevalence of 56%. The prevalence depends on the specific production discipline of the farm and ranges from 100% in breeding-to-farrowing to 20 % in farrowing-to-finishing herds, passing through 46.2% in purely breeding, 45.5% in purely farrowing and 69.2% in finishing farms. It seems that much more are movement of animals within a herd and much more is the possibility to acquire MRSA. Actually, it has been calculated a 11 times higher odds to be MRSA-positive for farms that have a MRSA-positive supplier. EFSA report (2009) had recognized in animal trading a risk factor for MRSA transmission between herds, so to indicate a top-down strategy as a chance for future control policy. Other remarks from Broens and colleague survey are related to antimicrobial use. Herds that use batch treatment are significantly more positive than herds that do not (OD=10.2; P=0.015). Moreover, resistance to tetracycline, erythromycin and clindamycin is a confirmation for CC398 in pigs. LA-MRSA is characteristically resistant to tetracycline; it is possible that the heavy use of this antibiotic in pig farming could be the cause of the emergence of such resistance. It has been demonstrated that the addition of tetracycline to animal feed increased the entity of nostrils contamination (Moodley et al., 2011). While resistance to ciprofloxacin is more frequently identified in poultry and veal isolates in Netherlands at least. The rate of contamination of pig farms is really high if in 58.5% of overall selected farms was found at least a dust sample positive for MRSA, with the highest frequency of detection in fattening ones (76.9%) and a slightly lower frequency (40.7%) in breeding and weaner to grower farms (Friese et al., 2012).

The contamination of pig farmers in Belgium was 37,8% in 2009, 86% in Germany (where in the same survey the 45% of veterinarians were found contaminated), 20% in Ontario (Canada). Differently from what happens in pigs, contamination in humans easily evolves often in infections, even severe and hard to treat such as abscesses, cellulitis and necrotizing fasciitis (Soavi et al., 2010; Lozano et al., 2011). LA-MRSA has demonstrated the ability to spread from

human to human out of the farm setting even if has a poor tendency to diffuse inside the hospital, perhaps thanks to its sensibility to non beta-lactam drugs.

The EFSA baseline survey found a strong association between positivity in breeding holdings and in production holdings following in the production chain. This is the result of the vertical or top-down diffusion of MRSA that should be interrupted or at least managed through prompt detection of positive herds. Typing of MRSA isolated should be routinely carried out in order to characterize and follow the evolution and diffusion of specific strains. Knowledge about MRSA strains circulating could help in managing outbreak in other species, including humans eventually infected through occupational exposure or food chain. The presence of MRSA in Italian pig herds and the specific types must be considered in the perspective of related dairy farms in high density livestock areas. The figures of the National Bovine Registry show that for instance in Lombardia region there are over 2,300 contexts in which bovines and pigs are reared in the same farm. Moreover, even if under different properties, the distance between farms could be as short as contaminant dust reaches dairy cows.

ZOONOTIC POTENTIAL AND OCCUPATIONAL EXPOSURE

The average MRSA carriage rate in general population is around 1% in several nationwide survey, even in countries characterized by a recognized heavy impact in animals (Jordan et al., 2011; Feingold et al., 2012). On the other hand some categories seems particularly at risk of being MRSA-positive. Actually, prevalence in these groups is surprisingly and significantly higher than in general population. Considering the profile of persons at risk it is easy to argue that their job could be a source of contamination. In The Netherlands rates of ST398 carriage among people working in contact with pigs and veal calves range between 25 and 35% (va den Broek et al., 2009). A European survey focused on human isolates from national laboratories found LA-MRSA ST398 in 8 out of 15 countries. The proportion of ST398 out of other MRSA lineages is under 2% in most cases, it is correlated to livestock density (pigs and cattle under 1 year age) and such a lineage is rare among blood infection, suggesting a lower virulence (van Cleef et al., 2011b). Owners and veterinarians of companion animals and occupational positions specifically linked to livestock farming (farmers, veterinarians, slaughterhouse workers) belong to categories at risk. The dynamic of contamination is particularly clear if the case of Meticillin-resistant *Staphylococcus pseudointermedius* (MRSP) in considered. MRSP is specifically a dog pathogen and so the reported prevalence of 3.9% among small animal dermatologists attending to a congress can be considered surprising. As *S. pseudointermedius* is not a common commensal of humans, this data represent an evidence of zoonotic transmission through exposure to patients with skin and soft tissue infections (Paul et al., 2011). The prevalence is major than that of MRSA (1.6%) and the data are consistent with those of a similar survey in the USA which found carriage rates of 5.3% and 3.5% for MRSP and MRSA respectively in 171 veterinary dermatologists (Morris et al., 2010).

Consulting international literature there's a general evidence of MRSA as a risk for veterinarians handling animals that support *S. aureus* infections. In Australia the general population MRSA carriage is about 0.7%, while evaluation of carriage among veterinarians takes the rate at higher levels, depending on prevalent area of work. Veterinarians attending to a conference in 2009 that accepted to be sampled by nasal swabbing were recruited in a survey aimed to determine differential risk of nasal carriage related to the prevalent activity. The percentage of veterinarians carrying MRSA in nasal cavities is higher than in general population (5.84%). Veterinarians operating in industry and government worked as control with a prevalence of 0.9%, without any difference relative to general population. Prevalence among small animal veterinarians was 5

times that of control (4.9%). Prevalence raised in a striking manner with veterinarians dealing with horses: 11.8% for those with horse among their patients and even 21.4% for those with horses as major activity (Jordan et al., 2011).

De Martino et al. (2010) found a low prevalence of MRSA in horses from 3 farms in Campania: 2 isolates in horses and none in people in contact with them. On the other side they found a relevant presence of Coagulase Negative Staphilococci resistant to meticillin (MRCNS), some of them indistinguishable from those isolated from staff members. Even if without involvement of MRSA, there's some evidence of cross-contamination between horses and humans in contact.

At what extent and for how much time a man could be contaminated by MRSA in a farm environment was investigated by Van Cleef et al. (2011). Thirty-four visitors were sampled by nasal swabbing for a total of 119 farm visits. The visit should consist of direct contact with animals. The 50 pig farms and 102 veal calves farms were sampled too to determine their MRSA status through nasal swabbing of animals and collection of environmental dust. Twenty-eight pig and 90 calves farms were positive. The human subjects were sampled before and after each visit and then again 24 hours later. None became nasal carrier after visiting a MRSA-negative farm. In 32 occasion a visitor became MRSA-positive but in 94% of cases in the 24 hours-later swabs he was decontaminated. Only one remained persistently a carrier. These data confirmed the high prevalence of MRSA in livestock in The Netherlands and the ease to be contaminated attending their environment. Such contamination likely derives from direct contact with animal carrying it in nares and inhaling dust. Contamination appears transient. Nevertheless, through this dynamic MRSA (potentially LA-MRSA) goes out from farms confines and enter the community.

There are different states of interaction between *S. aureus* and its host: infections, carriage or colonization, and contamination. MRSA can be persistently or intermittently carried by healthy humans, and colonization is the major risk factor for infection (EFSA, 2009).Transient or permanent MRSA carriage for categories at risk represent a potential tool to diffusion of the pathogen. They could transmit MRSA to their families (Huijsdens et al., 2006) and broader people exacerbating what is already occurring with CA-MRSA. The consequences could be an increased circulation of MRSA among general population and the risk of contamination of healthcare settings if members of categories at risk have to access to hospital. Another issue is the possibility that a veterinarian carrying MRSA could transmit it to their patients generating

new carriers and causing potential new pathologies supported by a *S. aureus* difficult to deal with.

In some countries with low prevalence of human MRSA infection, CC398 is a major contributor to the overall MRSA burden. In countries with high overall human MRSA prevalence, CC398 is considered of less significance for the public health (EFSA, 2009). Cases of severe deep-seated skin and soft tissue infections are reported due to ST398 MRSA likely acquired through occupational exposure to livestock (Soavi et al., 2010; Lozano et al., 2011). Where CC398 prevalence is high in food-producing animals, people in contact with these live animals (especially farmers and veterinarians, and their families) are at greater risk of colonization and infection than the general population (EFSA, 2009). Food may be contaminated by MRSA (including CC398): eating and handling contaminated food is a potential vehicle for transmission too. Nevertheless, there is currently no evidence for increased risk of human colonization or infection following contact or consumption of food contaminated by CC398 both in the community and in hospital. Otherwise, MRSA (including CC398) can enter the slaughterhouse in or on animals and occur on raw meat. The primary reservoirs of CC398 in affected countries are pigs, veal calves, and broilers (EFSA, 2009).

There's the need that further work should be performed on harmonizing methods for sampling, detection and quantification of MRSA carriage in both humans and animals, as well as for detection of MRSA as a contaminant of food, and in the environment including from dust both in air and on surfaces. Much knowledge should be acquired at this regard an much attention should be paid in prevention of MRSA spreading in settings recognized at risk.

PART I:
SPREAD OF MRSA IN TWO FARMING CONTEXTS WITH DAIRY COWS AND PIGS AT CLOSE CONTACT

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important concern for human health. The worldwide emergence of MRSA and the raising awareness of health as a unique concept concerning both humans and animals are important issues nowadays. The willingness to prevent zoonoses enforces this point in a world without boundaries. International travelling and trading of goods and animals promote diffusion of pathogens beyond local extent (Févre et al., 2006; van Loo et al., 2007; Stenhem et al., 2010). Moreover tracing strains pathway of spreading takes advantage of more and more new scientific achievements thanks to molecular biology. In Europe, especially in The Netherlands and Denmark, the papers about MRSA prevalence and its diffusive strategies even among production animals are really abundant. They well-describe the remarkable contamination of production animals (in special extent veal calves, pigs and poultry) and the potential amplification of diffusion among particular occupational categories and consequently to their families and the community. People living in rural area are more at risk to be LA-MRSA carrier than people living in urban area, even without direct contact or any epidemiological link with livestock (Feingold et al., 2012). The same contaminant pressure is probably reciprocal among production animals species, that is large size pig holdings more likely to be MRSA-positive (Battisti et al., 2012) could be a source of contamination for dairy farms. Live animals trading and transport are clearly factors able to heavily influence infections epidemiology through strains circulation and cross-contamination, beyond species-specificity and beyond national borders. Still too little is known about MRSA importance for animal in Italy.

S. aureus has a strong impact on health and production. The occurrence of MRSA in dairy herds could reveal a further challenge issue in *S. aureus* control and a threat to workers health too (Soavi et al., 2010). As a major pathogen, udder infections due to *S. aureus* challenge dairy industry to be economically rewarding. When control by biosecurity fails, therapy becomes a chance to rescue an animal or an udder quarter. Therapy often results disappointing, even with the evidence of encouraging antimicrobial susceptibility test results. Some opinion makers consider a cow positive to *S. aureus* infected for life (Barkema et al., 2006). Drug administration constitutes a cost and is not advisable in presence of strains with recognized specific resistance patterns (van den Borne et al., 2010). Resistance to β-lactams conferred by the *mec*A gene

encoded Penicillin Binding Protein (PBP2) suggests to give up treating mastitis and increases the rate of culled cows.

Awareness about real prevalence and risk factors is necessary for prevention. Recently an unexpectedly high prevalence (9.3%) of ST398 in Belgian dairy herds was revealed based on (Vanderhaghen et al., 2010). Estimation by bulk tank milk samples determined a 4.4% prevalence in Germany and all the isolates belonged to CC398 (Kreausukon et al., 2012). Still in Germany, Spohr (2010) investigated isolates from milk and other specimens within three MRSA-positive dairy farms. Nevertheless in the latter paper only the largest size farm has in anamnesis an epidemiological relation with a pig farm and the transmission pathway was not demonstrated. Although epidemiology of MRSA in bovine mastitis could be more complex and involve different lineages commonly seen in humans in hospital and community settings (Türkyılmaz et al., 2010), relevant pigs presence represents a major risk factor for dairy herds.

The lack of data about the weight of MRSA in dairy cows of our territory and the awareness of importance of *S. aureus* infection control make the strength to investigate its presence to raise. The aim was to identify MRSA in subclinical and clinical mastitis cases which could be a threat to therapy and so to control of infection within an herd. The search began screening *S. aureus* isolates from current diagnostic service to dairies. Resistance to oxacillin was used as selective test to be eventually confirmed by PCR. In brief two positive herds were identified and it was surprising that both were in strict spatial relation with a pig holding of relevant size. Such a relation was not found in negative herds. An epidemiological survey became necessary to search for the real source of infection and the risk for humans too. This chapter describes the screening methods and the searching for MRSA in the two positive farms and related environment. Isolates were characterized at molecular level in order to collocate them among circulating strains according to animal species and possibly geographical area. An attempt has been made to define the pathaway of contamination/infection in each farming system to allow the farmer to keep on working in biosecurity.

MATERIALS AND METHODS

MRSA identification in milk

All isolates from subclinical and clinical cases of mastitis identified as *S. aureus* were considered. All milk samples were processed and pathogen identified according to NMC guidelines (NMC, 1999). The isolates came from current daily diagnostic activity of the laboratory service to the farms from February to June 2010. The isolates of Gram-positive cocci, oxidase and catalase positive, confirmed by coagulase-positive test as *S. aureus* were streaked onto Mueller-Hinton agar added with 6 µg/ml oxacillin (Corrente et al., 2007) as screening method. Resistant strains faced a second step testing their susceptibility to cefoxitin (30 µg disc content; Biomeroieux, Mercy d'Etoile, France) by agar diffusion method. A halo diameter <19 mm means resistance. Definitive confirmation of MRSA was performed by a duplex PCR adapting a protocol for *mec*A gene (Murakami et al., 1991) and *nuc* gene (Baron et al., 2004) amplification in a single reaction. *Staphylococcus aureus* ATCC 33592 was included in each reaction as control strain.

Collection of supplementary specimens in MRSA-positive farms

In consideration of the importance and epidemiology of MRSA in humans and animals, a positive dairy farm had to be deeply investigated. Nasal swabs (Copan, Brescia, Italy) from workers (N=6) were collected on farm by a Surgeon. The author collected nasal and vaginal swabs from cows (N=15) on farm and nasal swabs from pigs (N=33) at the slaughter-house. All the swabs were kept in Amies transport medium at 4°C until processing. Environmental specimens consisted in dust from pigs facilities collected by a sterile gauze (N=6) and air (N=5) absorbed by the SAS device (30 L per plate), the latter used only in farm A. The gauzes were kept in a sterile plastic stomacher bag until analyses.

Microbiological processing of specimens

Each swab underwent two successive enrichment steps. They were firstly inserted in a tube of 9 ml Mueller-Hinton broth added with 6.5% NaCl and incubated at 37°C for 18-20 h. Then 1 ml transferred in new tubes of Tryptic Soy Broth added with 3.5 mg/l cefoxitin and 75 mg/l aztreonam and incubated for other 18–20 h at 37°C. One hundred µl of the final resulting broth was plated onto MRSA Chromogenic Agar (Pronadisa, Madrid, Spain) and incubated in the same condition and time. The same procedure was followed for dust samples. At reading, at least

five blue-greenish colonies was isolated on blood agar as putative MRSA. The isolates were then streaked onto Mueller-Hinton agar added with 6 µg/ml oxacillin. For confirmation the oxacillin-resistant isolates were inoculated in Brain Heart Infusion and after incubation the DNA was extracted as described by (Cremonesi et al., 2006). A duplex Polymerase Chain Reaction targeting *nuc* (Baron et al., 2004) and *mec*A (Murakami et al., 1991) genes was performed.

Blood agar and Baird Parker agar plates inoculated with air by SAS were incubated and read at 24 and 48 h respectively. Suspected colonies were isolated and isolates investigated like those from milk and swabs.

All the isolates were stored in Nutrient Broth added with 15% glycerol at -80°C till the moment of the following analyses.

Identification of virulence genes

The DNA was amplified to verify the presence of virulence-associated genes using primers and protocols described in literature. The virulence factors considered were thermonuclease encoded by *nuc* gene, and the enterotoxins encoded by *sea, sec, sed, seg, seh, sei, sej, sek, sel* (Cremonesi et al. 2005); other two enterotoxins gene (*seb, see*) were amplified with a different set of primers (Pinto et al. 2005 and Monday et al. 1999). *spa* and *coa* genes, coding for the surface protein A and coagulase respectively, were used to type the isolate on the basis of length polymorphism of the amplimers; *clf*A (clumping factor A), *tsst* (toxic shock syndrome toxin 1), *eta, etb* (exfoliatin toxins A and B): all these genes were targeted with the primers published by Akineden et al. 2001. We also searched for leukocidin E *leuk*E (Fournier et al. 2008); LukS-LukF/PV also known as Panton-Valentine Leukocidin, *mec*A (McClure et al. 2006); *sak* (encodes an anti-opsonin and inhibitor of defensins staphylokinase), *fmtB* (a gene encoding a cell-wall-associated protein), *scn* (staphylococcal complement inhibitor), *chp* (chemotaxis inhibitory protein) (Sung et al. 2008); the gene coding for LuKE-LukD and LukM leukocidins (Jarraud et al. 2002); collagen binding protein, *cna* (Zecconi et al. 2006).

Multi Locus Sequence Typing, spa-typing

MRSA isolates were genotyped by DNA sequencing of the X-region of the Staphylococcus Protein A (spa-typing, Harmsen et al., 2003), with repeats and spa-types determined by Ridom StaphType software (Ridom GmbH, Würzburg, Germany) and Multi-Locus Sequence Typing (MLST) (Enright et al., 2000).

RS-PCR

For RS-PCR genotyping, the method rearranged by Fournier et al. (2008), was used. This method is based on the amplification of the 16S-23S rRNA intergenic spacer region. In a total volume of 25 µl, each reaction contained 1x HotStarTaq Master Mix (Qiagen), 800 nM of each primer (G1:GAAGTCGTAACAAGG and L1:CAAGGCATCCACCGT) and ~30 ng of DNA. The PCR protocol was: 95 °C for 15 min, followed by 27 cycles comprising 94 °C for 1 min, followed by a 2 min ramp and annealing at 55°C for 7 min. After a further 2 min ramp, extension was done at 72 °C for 2 min. PCR products were then analyzed using an Agilent 2100 Bioanalyzer with a DNA 7500 LabChip kit (Agilent Technologies, Palo Alto, CA). For interpretation of the results, two patterns were considered different if two or more peaks of the electropherogram differed in size. Grouping of the RS-PCR profiles was obtained with the BioNumerics 5.0 software package (Applied Maths) using the UPGMA (Unweighted Pair Group Method using arithmetic averages) cluster analysis.

Antimicrobial resistance pattern

The MRSA isolates were tested for antimicrobial susceptibility determined by the disk diffusion method, according to CLSI guidelines (2008). The panel included drugs used for mastitis therapy and molecules representative of all antimicrobial classes: amoxicillin/clavulanic acid (AMC, 20/10 µg), ceftiofur (CEF, 30 µg), cefoperazon (CFP, 30 µg), cefquinome (CEQ, 10 µg), penicillin G (P, 6 µg), marbofloxacin (MAR, 5 µg), danofloxacin (DFX, 5 µg), ciprofloxacin (CPR, 5 µg), tetracycline (TE, 30 µg), gentamicin (GM, 10 µg), kanamycin (K, 30 µg), sulphametoxazole/thrimetoprim (STX, 1.25+23.75 µg), vancomycin (VAN, 30 µg).

Statistical analysis

Fifty two isolates for which the results of MLST, *spa* typing, RS-PCR and *spa* length polymorphism were all available were used to calculate Simpson's Diversity Index that is a measure of diversity which takes into account the number of types present, as well as the relative abundance of each type. Calculation of Simpson's diversity index was performed based on the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

where *N* is the total number of isolates in the sample population, *S* the total number of genotypes described, and *nj* is the number of isolates belonging to the *j*th genotype. The value of ***D*** ranges between 0 and 1. With this index, 1 represents infinite diversity and 0, no diversity (Hunter et al., 1988).

RESULTS

The farms

In five months observation 183 *S. aureus* isolates were screened from 27 dairy farms. Thirty-three isolates from three farms (A, B and C) was confirmed MRSA. An epidemiological survey on each farm situation highlighted the presence of pig holding of relevant size nearby the farms. Farms A and B was characterized by a particularly strict connection between the two species without a safety distance in the regards of biosecurity. Whereas farm C is localized in a municipality with 12 pig holdings still active (from 2001 to 2009 other 9 ceased activity). Out of 12, seven are fattening holdings (consistency of 700, 3750, 1150, 2000, 1860, 1107 and 3001 pigs respectively), and for the remaining consist of few pigs for home-consumption. So, this farm is surrounded by potentially contaminating livestock through waste releases or air but with a minimum distance from them which lacks is A and B farming systems. For this reason we investigated these latter more deeply.

Farm A reared dairy cows and pigs under the same property. The dairy consists of 180 dairy cows reared in free-stall facilities. The part of the farm dedicated to pigs is a farrow-to-finish with a total of 3,135 pigs. The staff is constituted by the two owners and some employees. One owner (1) manages the dairy and milks twice daily helped by another employee milker. The other owner (2) is the pigs caretaker but is involved in all activities within the family property, including daily milking operations.

Farm B owns only the dairy cows (N=50). The pig holding is a fattening one and is part of a production chain where the pigs are held only for the finishing period before slaughtering. The pig holding consists of five sheds harboring about 4,000 fattening pigs. The property is other than that of cows but the pigs caretaker and the owners of dairy cows have free access to the whole area with no confinement. The farmers (father and son) milk twice daily their cows. They don’t use gloves and applied a post-dipping product.

Environmental, swine and humans specimens

In farm A none of the tested workers (N=2), included the employee milkman, was found carriers of MRSA. The owners 1 and 2 were MRSA positive for nasal carriage. Six colonies from each plate were collected and isolates were characterized as displayed in Table 1. Two pigs were sampled on farm visit, one was MRSA positive. Thirty-three out of a batch of 130 pigs were sampled at slaughter, from 15 MRSA was isolated. Dust collected by sterile wipes from shed was positive too, whereas SAS method was abandoned for pigs facilities because of the excessively dusty air which determined a quick filling up of the plate with too many overlapping colonies. In cows facilities air sampling through SAS was feasible but only MRCNS were recovered.

In farm B both owners resulted negative for MRSA nasal carriage. Nasal and vaginal swabs were collected from fifteen lactating cows and a nasal swab from the dog. In none MRSA was isolated. On the other hand, environmental dust from each sheds of fattening pigs and from milking parlor facilities resulted widely harboring MRSA.

MLST, spa typing, RS-PCR, spa polymorphism and Simpson's Index

The results of MLST, *spa* typing, RS-PCR and *spa* gene length are all displayed in table 2 and 3. A synthesis of the reliefs and specific lineages detected in each species involved is proposed for farm A in figure 4 and for farm B in figure 5. A dendrogram in figure 3 show the homology between the isolates analyzed. The Simpson's Diversity Index calculated on the 52 isolates for which the results all the techniques applied were available resulted in a decreasing order: 0.45 for MLST, 0.30 *spa* typing, 0.15 for RS-PCR and 0.05 for spa length polymorphism. These data confirm MLST as the most discriminating methods and the *spa* gene length polymorphism as the less able to found differences between *S. aureus*.

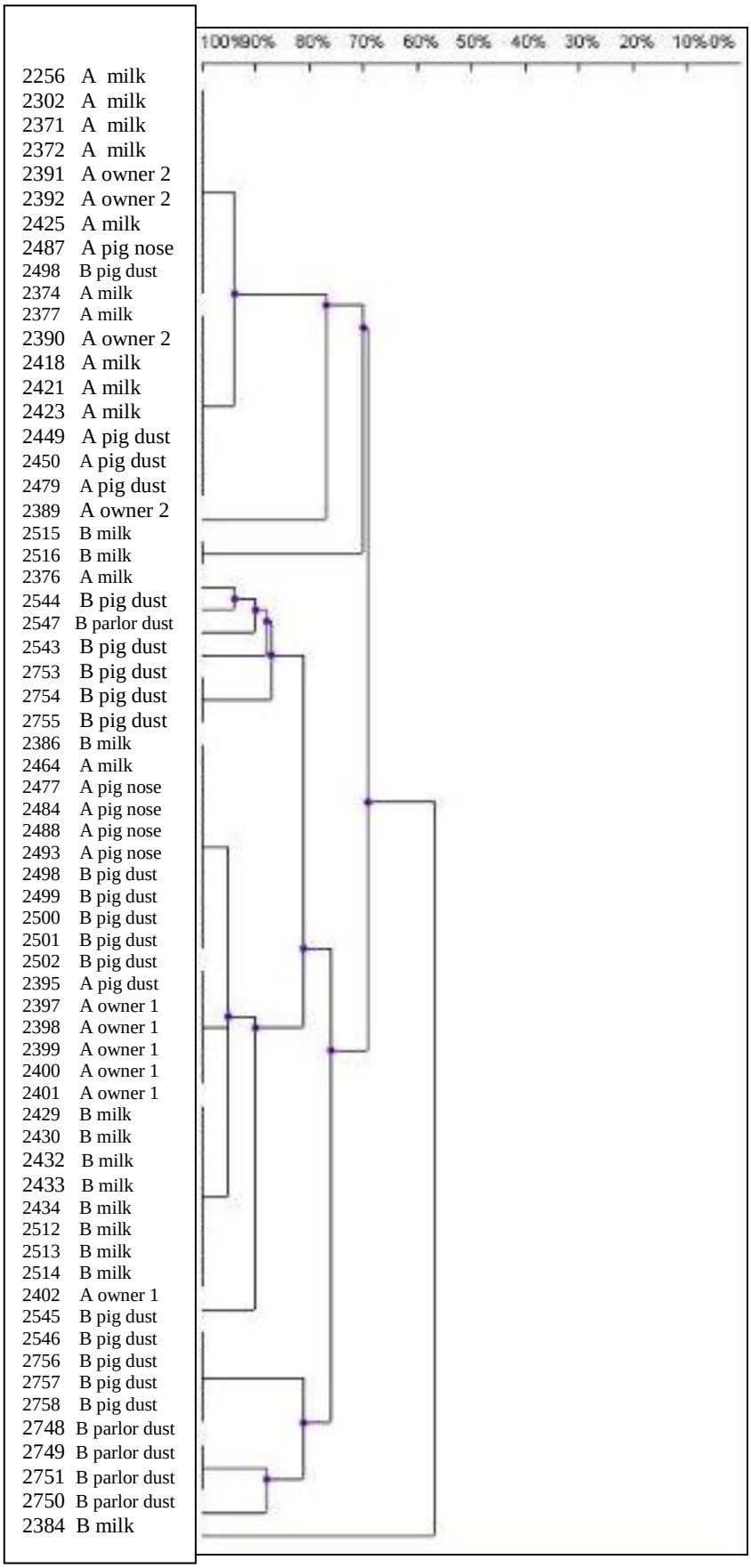

Figura 3: dendrogran obtained by RS-PCR evaluation of isolates from both farm A and B and from different matrices. The tree branches represent the homology degree between isolates. ID and description are those of table 3 and 4.

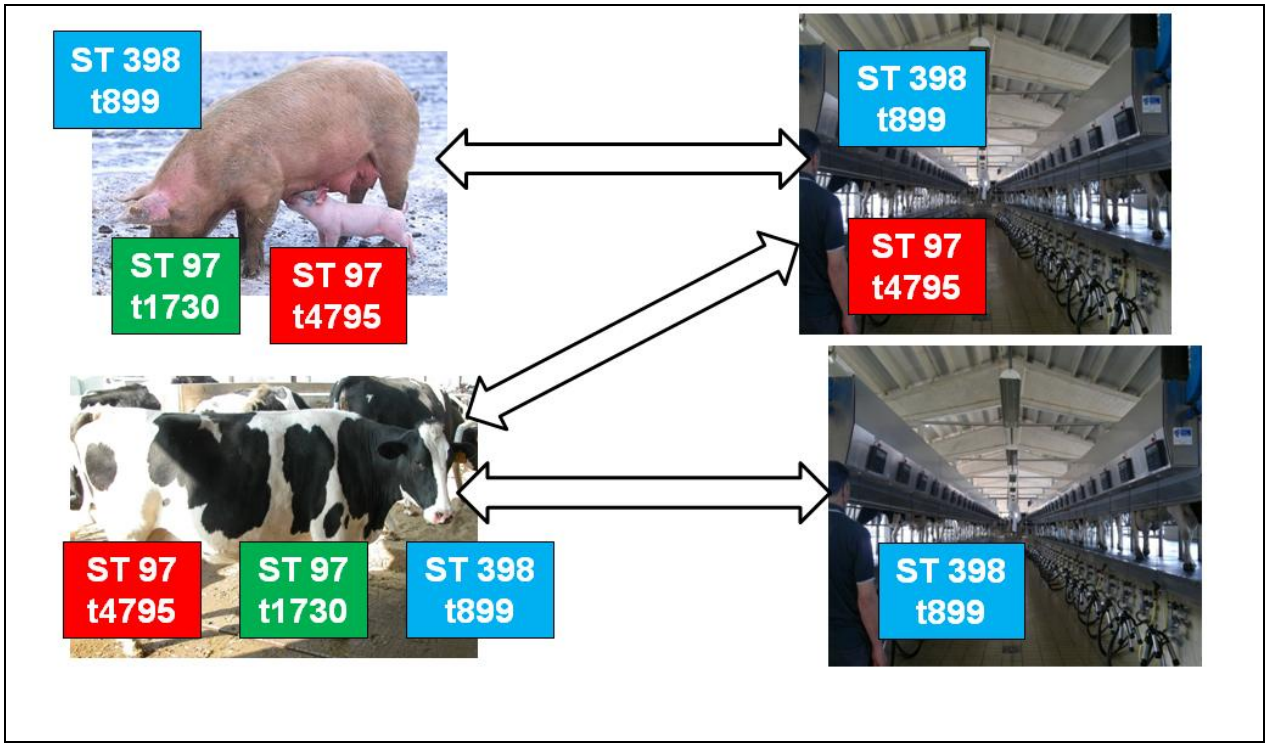

Figura 4: synthesis of lineages identified in farm A.

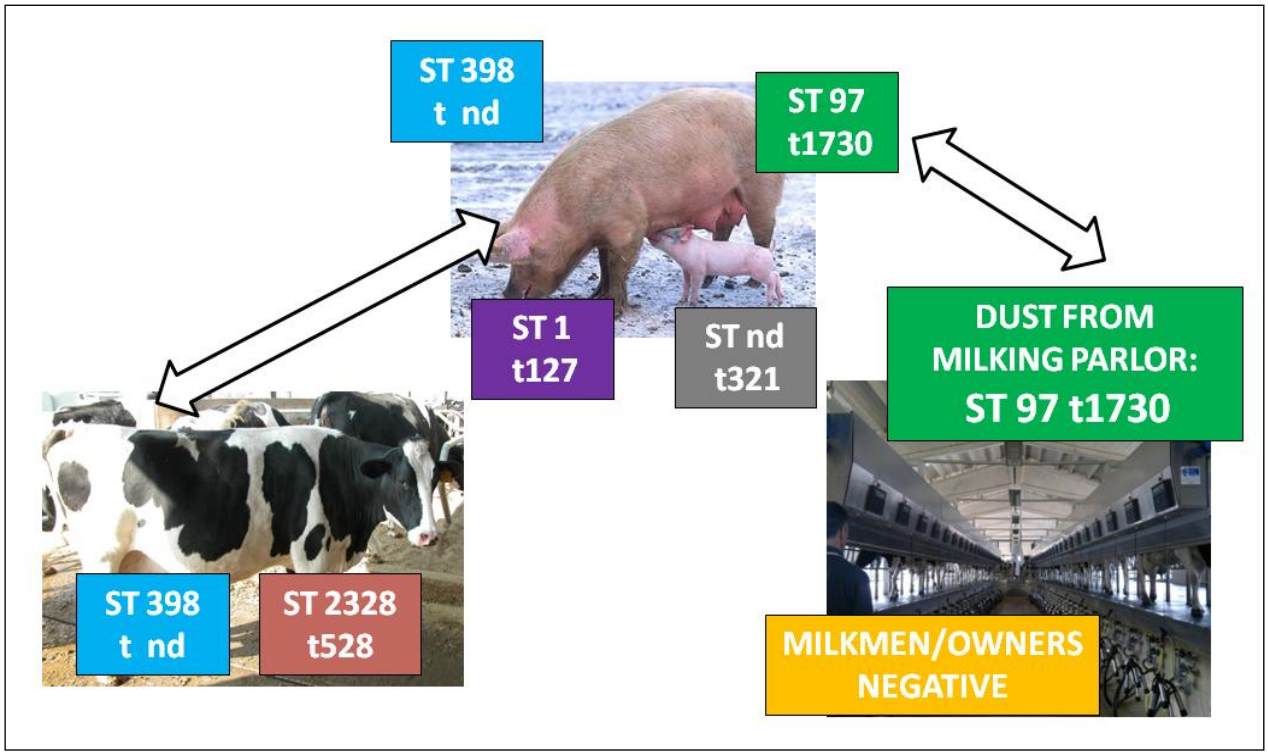

Figura 5: synthesis of lineages identified in farm B (presuming that the spa type in pigs and milk is t899, like in farm A).

Virulence genes

Most isolates were negative for enterotoxins but nine isolates harboring *she* gene belonging to three different lineages (ST398, ST1 and ST97) in farm B from the dust of two pigs sheds and one from milking parlor dust. Isolate 2758 from a pig shed in context B resulted positive for A and J enterotoxins. All MRSA were positive for clumping factor A (*clfA*) but fifteen ST398 isolates: two from owner 2, two from pigs noses in context A; seven form milk and four from pig facilities in context B. All isolates resulted positive for *leukE* and *cnapicc*. All MRSA were negative for exfoliative toxins (ETA and ETB). About half of isolates were positive for PVL-ED, representing almost all lineages revealed, whereas all were negative for PVL-M. Four ST398 MRSA from milk in farm B and one ST97 t1730 from a pig swab in farm A were positive for *sak* gene. Most isolates carried *fmtb* gene, with the exception of a 10, three ST97 t4795 from bulk tank milk and two ST97 t1730 from pig swabs in farm B, four form milking parlor dust in farm B belonging to the same lineage. The human isolate 2389, a ST398 from owner 2 in farm A harbored the gene *tsst* coding for shock syndrome toxin 1 and *scn*. All MRSA were negative for *chp*.

Antimicrobial susceptibility

Fourteen isolates representative of the lineages isolated in different matrices in the two farms were considered. All the tested isolates were resistant to TE and P, none was VAN resistant. Two isolates belonging to ST97 t1730, one (2256) from milk in farm A and one (2747) from dust sampled in milking parlor in farm B, were resistant to STX. Isolates 2256 and 2747, together with 2377 from milk in farm A (ST97 t4795), 2450 from a pig nasal swab in farm A (ST97 t1730) and 2753 from dust in pig shed in context B (ST97 t1730) were also resistant to fluoroquinolones. About aminoglycosides, susceptibility seems to be conserved, but in isolate 2747 (ST97 t1730), from the dust of the milking parlor in farm B, that was resistant to gentamycin and kanamycin. Susceptibility to cephalosporins seems partially conserved but for cephoperazon

DISCUSSION

MLST and *spa*-typing of MRSA from farm A and B confirms the main lineages present in pigs in Italy where a specific *spa*-type distribution has been observed (EFSA, 2010). The most prevalent *spa*-type in Italy t899 (ST398) was found in each area of epidemiological survey in farm A (milk, dust from pigs facilities and humans nasal swabs). Also in farm B ST398 t899 was isolated in milk and dust from pigs, but not in humans or cow environment. There's the evidence of the wide circulation of MRSA within both contexts and at the same time that the likely origin are the pigs. Surely this is a clear demonstration of pigs responsibility as the source of contamination, together with the remark that no farm with *S. aureus* isolates negative to the screening had strict relation with pig holdings. Moreover ST398 t899 ubiquity is an evidence of the geographical specificity in spatial distribution of molecular types. The other lineages identified are well known in Italian pigs holdings too (Battisti et al., 2010). ST97 t1730 was found in farm A in milk and pigs but not in humans, and in farm B in pigs and dust from milking parlor. ST97 MSSA has already been associated to bovine mastitis displaying a resistance profile more relevant than other animal isolates (Sung et al., 2008). Even if it was not recovered in milk from farm B cows, MRSA ST97 found in dust from milking parlor, likely coming from pigs area e.g. through wind (Friese et al., 2010), could exert a contaminating pressure and eventually infect udders during milking. It suggests a potential source of infection from the environment (Capurro et al., 2010) with or without direct contact with milkers' hands. A specific risk factor in farm B could be that the owners didn't wear gloves during milking. ST1 t127 is a non-ST398 typically Italian MRSA (EFSA, 2010; Franco et al., 2011) and confirmed in this work in pigs facilities but never recovered among the milk isolates. A persistent udder infection sustained by non-ST398 MRSA ST1 t127 has been described in one cow in Italy (Pilla et al., 2011), so it likely that also this lineage could infect cows.

The analysis on human isolates from nasal swabs revealed that both the owner of farm A are exposed to the risk of being carrier of MRSA typically present in Italian pigs and demonstrated in their own pigs too. While the employee milker was negative, owners 1 and 2 were both positive and this could be the mirror of the different frequency and density of exposure to a shared contaminated environment. The owners work all day long in the farm and live nearby, so the weight of exposure is higher and similar irrespective to the specific individual main role in farm management. Nevertheless, owner 1 harbors apparently only ST398 t899, while owner 2 at the swabbing moment carried both ST398 t899 and ST97 t4795, a lineage recently recovered and

firstly identified in pigs in Italy (Battisti et al., 2010). The variety of lineages recognized is a confirmation of the heterogeneity of MRSA among Italian pigs. Such a variety reflects also in lineages recovered in milk, suggesting a dependence on several successive introduction of strains over time which resulted in a pool of diverse strains. The employee milker might be less exposed as his activity consists mainly in milking procedures in contact with cows but not with pigs, and at a lower extent compared to the owners. The owners of farm B harbored no MRSA and only MSSA were isolated. The difference between contamination and steady carrier status represents a fundamental epidemiological issue. In both cases a human could transmit MRSA to other people and to animals through direct contact as a nasal carrier is easily a hand carrier too. The duration of MRSA carriage conditions the time at risk of further spreading. Some professional categories such as veterinarians, farmers and slaughter workers has been demonstrated to be particularly at risk of become MRSA-positive through exposure to animals and their environment (van Loo et al., 2007). Among these categories MRSA prevalence is significantly higher than in general population (Feingold et al., 2012; Jordan et al., 2011; Loeffler et al., 2011). Field workers exposed to intensive physical contact with animals and sampled before and after visiting a MRSA-positive veal calves or pigs farm are at high risk to acquire the same *spa* type of that farm. Nevertheless, if sampled after 24 hours in 94% of cases they are negative again (van Cleef et al., 2011). This might justify the negativity of the farm B owners who although chronic exposure could be transient carriers and have been sampled in a moment of MRSA clearance. Moreover, negative nasal swabs has been reported in personnel working in a pig farm and living nearby (Lozano et al., 2011). MRSA clearance might also justify the absence in farm A owner 1 of all the strains revealed in owner 2 and probably coming from the same environment. As positivity of people working with animals can be due to repeated contamination rather than to chronic colonization, the MRSA population in nasal cavities of the same worker can be heterogeneous and different from sampling to sampling, or even absent.

The potential to acquire MRSA can be conditioned by species-specificity of strains (Sung et al., 2008) or to adaptation to host species (Price et al., 2012). A recent sequence-based comparison among field isolates of CC398 produced data suggesting that this lineage was previously tipically human, successively transmitted to animals losing genes for human-niche and adapting to non-human hosts. Then it acquired *mec*A and other resistance genes becoming MRSA. The costs of these evolutionary dynamics are lower virulence and rate of spreading, specially towards humans (Price et al., 2012).

Otherwise, the behavior at work, that is the adoption of hygienic practices and the use of appropriate personal protective equipment, can be crucial too (van Cleef et al., 2011). The use of gloves in animal handling is always suggested and during milking it represents a cheap and effective way to prevent transmission of contagious pathogens like *S. aureus*. The variety of sources in which MRSA was isolated in this work suggests a composite route of infection for cows. In farm A three different strains was found in milk and this could mean a spreading dynamic other than merely contagious. The most frequently isolated strain was ST97 t4795, also found in bulk tank milk, so to be considered the dominant one. ST398 t899 was isolated in only a mastitis case. For these two strains carriage by owner 1 and 2 was demonstrated, suggesting both their status of vector or a similar exposure level to contamination for humans and dairy cows. ST97 t1730 was not revealed in humans but demonstrated able to induce mastitis and was widely present in pigs noses and presumably in dust as well as the other strains. In farm B ST398 t899 is the only MRSA isolated in milk, with only an exception for ST2329 t598 in one sample, suggesting a purely contagious way of transmission. The milkers didn't wear gloves which are considered an important tool for prevention of contagious pathogen diffusion from cow to cow. A human vector was not identified at time of swabs collection, but environment was demonstrated contaminated by MRSA. In particular, dust collected from milking parlor was found reach of ST97 t1730, a strain revealed in pigs shed 2 dust but not in milk. The position of the milking parlor in relation to the pig sheds can mean an open door to the shift of MRSA from pig context to dairy cows. Like human nose population, the pool of MRSA strains present in dust can change in time justifying the lack of a direct link with milk isolates at the time of sampling. Thus, considering the instant of sampling in each farms a snapshot of situation, one could argue that in farm A both the owners could be a link between pigs and cows. Otherwise, in farm B the owners, though negative at present, could have harbored transiently MRSA strains found in milk samples. The milking environment represents a source of other potentially infective strains in farm B, while the environment in general in both context A and B is a reservoir of strains likely coming from pigs due to brief distance and wind carriage (Gilchrist et al., 2007; Friese et al., 2012).

Antimicrobial susceptibility patterns of tested isolates demonstrated to be a marker for MRSA. All confirmed resistance di tetracycline, a feature really typical, especially in LA-MRSA, perhaps because of exposure to batch therapy with these drug class. Resistance to penicillin and to other beta-lactams (cephalosporin), even with different extent, gives an idea of highly limited

possibilities of cure that MRSA offers in mastitis therapy. Penicillin resistance itself means a negative prognosis on cure rate. Otherwise, resistance to florquinolons is typical of non ST398 lineages, that's to say that it is present in lineages that, at least in origin, had been exposed to species in which florquinolons are used (poultry, humans …). LA-MRSA specific of pigs are susceptible to this antimicrobial class. Resistance to sulfammide/thrimetoprim seems associated to ST97 t1730 and to resistance to aminoglycosides, as these antibiotics are efficacious on any other lineages.

CONCLUSION

Strict relatedness between dairy farms and large pig holdings seems to constitute a risk factor for lactating cows to experience mastitis caused by MRSA. The suspect is supported by the remarks of the epidemiological survey about the two farms in the present work. Although further investigation on large scale should be done to confirm and quantify such a risk. Molecular characterization of isolates in each compartment and their comparison suggest that there's an active and dynamic sharing of MRSA lineages between species and places. As *S. aureus* and even MRSA is not a problem in pigs industry, the health threats are zoonotic risk for humans (consumers and workers) and mastitis for cows. A safety distance is advisable between any type of reared livestock and others. Clearly it is not conceivable to take quickly a farm away from another in field reality. The only cost-effective policy is to prevent udder disease due to MRSA. Measures to achieve it are the same for *S. aureus*, in particular hygienic milking practices to be enforced in consideration of the potential environmental pressure.

Table 2 Source, description and molecular identification and classification of MRSA from farm A

ID	Context	Origin	Description	spa Type	ST	RS-PCR	spa length
2256	A	milk	mastitis case	t1730	97	A	>250
2302	A	milk	mastitis case	t1730	97	A	>250
2371	A	milk	mastitis case	t4795	97	A	>250
2372	A	milk	mastitis case	t4795	97	A	>250
2374	A	milk	mastitis case	t4795	97	A'	>250
2376	A	milk	bulk tank milk	nd	nd	nd	Nd
2377	A	milk	mastitis case	t4795	97	A'	>250
2389	A	human, nasal swab	owner 2	nd	398	nd	>250
2390	A	human, nasal swab	owner 2	t4795	97	A'	>250
2391	A	human, nasal swab	owner 2	t4795	97	A	>250
2392	A	human, nasal swab	owner 2	nd	nd	A	>250
2395	A	dust	fattening shed	t899	398	C	>250
2396	A	dust	fattening shed	t4795	97	A	>250
2397	A	human, nasal swab	owner 1	t899	398	C	>100
2398	A	human, nasal swab	owner 1	t899	398	C	>100
2399	A	human, nasal swab	owner 1	nd	398	C	>100
2400	A	human, nasal swab	owner 1	nd	398	C	>100
2401	A	human, nasal swab	owner 1	nd	398	C	>100
2402	A	human, nasal swab	owner 1	nd	398	nd	>100
2418	A	milk	bulk tank milk	t4795	97	A'	>250
2421	A	milk	bulk tank milk	t4795	97	A'	>250
2423	A	milk	bulk tank milk	t4795	97	A'	>250
2425	A	milk	bulk tank milk	t4795	97	A	>250
2449	A	pigs, nasal swab	at slaughter	t1730	97	A'	>250
2450	A	pigs, nasal swab	at slaughter	t1730	97	A'	>250
2464	A	milk	mastitis case	nd	398	B	>100
2477	A	pigs, nasal swab	at slaughter	nd	398	B	>100
2479	A	pigs, nasal swab	at slaughter	t4795	97	A'	>250
2484	A	pigs, nasal swab	at slaughter	t899	398	B	>100
2487	A	pigs, nasal swab	at slaughter	t1730	97	A	>250
2488	A	pigs, nasal swab	at slaughter	nd	398	B	>100
2493	A	pigs, nasal swab	at slaughter	nd	398	B	>100

Table 3 Source, description and molecular identification and classification of MRSA from farm B

ID	Context	Origin	Description	Spa Type	ST	RS-PCR	spa length
2384	B	milk	mastitis case	t528	2328	nd	100
2386	B	milk	mastitis case	nd	398	nd	>250
2429	B	milk	mastitis case	nd	398	D	>250
2430	B	milk	mastitis case	nd	398	D	>250
2431	B	milk	mastitis case	nd	398	D	>250
2432	B	milk	mastitis case	nd	398	D	>250
2433	B	milk	mastitis case	nd	398	D	>250
2434	B	milk	mastitis case	nd	398	D	>250
2498	B	dust	shed 5	nd	nd	A	>250
2499	B	dust	shed 5	nd	398	B	>100
2500	B	dust	shed 5	nd	398	B	>100
2501	B	dust	shed 5	nd	398	B	>100
2502	B	dust	shed 5	nd	398	B	>100
2512	B	milk	mastitis case	nd	398	D	>100
2513	B	milk	mastitis case	nd	398	D	>100
2514	B	milk	mastitis case	nd	398	D	>100
2515	B	milk	mastitis case	nd	398	D	>100
2516	B	milk	mastitis case	nd	398	D	>100
2543	B	dust	shed 4	t321	nd	nd	>100
2544	B	dust	shed 4	t321	nd	nd	>100
2545	B	dust	shed 4	t321	nd	E	>100
2546	B	dust	shed 4	t321	nd	E	>100
2747	B	dust	milking parlor	t1730	97	nd	>100
2748	B	dust	milking parlor	t1730	97	F	>250
2749	B	dust	milking parlor	t1730	97	F	>250
2750	B	dust	milking parlor	t1730	97	nd	>250
2751	B	dust	milking parlor	t1730	97	F	>250
2753	B	dust	shed 2	t127	1	G	>250
2754	B	dust	shed 2	t1730	97	G	>250
2755	B	dust	shed 2	t127	1	G	>250
2756	B	dust	shed 2	t127	1	E	>250
2757	B	dust	shed 3	nd	398	E	>100
2758	B	dust	shed 3	nd	398	E	>100

PART II:
BULK TANK MILK AND GEOREFERENTIATION AS EPIDEMIOLOGICAL TOOLS

INTRODUCTION

Direct contact is considered the main route of diffusion for *S. aureus* and so should be for MRSA as well. We discussed above the pathogen spreading potential of environmental dust in two dairy farms each rising in strict proximity to a pig holding. We demonstrated the presence of LA-MRSA and other MRSA lineages both in dust (from pigs facilities, pigs nares, milking parlor facilities) and in milk. Moreover, MLST and spa-typing confirmed the cross-contamination of the two farming system, the dairies and the pig holdings. In general, a bidirectional transmission humans-animal is likely. Individual cows could become infected both classically through milkers hands and through environmental items contaminated by dust (Capurro et al., 2010). The milkers, at least in farm A, were found MRSA nasal carrier and it is known that there's a positive correlation between nasal carriage and MRSA presence on hands (Leonard and Markey, 2008).

For humans, proximity to farms is a demonstrated risk factor, even in absence of direct contact. A recent study (Feingold et al., 2012) showed that 44.4% of the subjects without direct contact with pigs or cows were LA-MRSA positive. They calculated an odds ratio of 11.2 for people leaving in rural area against people leaving in urban settings. They argued that regional density of livestock could be a risk factor for general population, not only for occupational categories (veterinarians, farmers, slaughterhouse workers), to be LA-MRSA nasal carrier. Similarly a correlation between LA-MRSA ST398 and pigs and cattle population density was unexpectedly observed (van Loo et al., 2007).

MRSA is a public health concern and has become a livestock challenge as well. Risk is to be intended not only in the humans regards but is reciprocal among production animal species too. In consideration of MRSA prevalence in pigs, EFSA report (2010) conclusions and the remarks of farm A and B cases in this work, we can presume that pigs represent a notable source of contamination for dairy cows. In absence of epidemiological evident links (shared personnel, movement of people and means etc.), risks can derive from brief distance between farms or from population density. It is likely that animal burden (number, type and size of herds, entity of animal movement) could be a factor conditioning the infective pressure on negative herds.

Bulk Tank Milk (BTM) represents a good and irreplaceable epidemiological tool in dairy farms. Total and differential bacterial counts of BTM provide an idea of hygienic level of the herd as a mirror of cleanliness score and preparation of the udder to milking. On the other hand and even

more important, BTM in the key to obtain by a single sample information about presence among lactating cows of pathogens detectable through culture, molecular biology and serology techniques. Bacteriological analysis is daily applied to BTM to detect contagious udder pathogen in order to improve management practices aimed to eliminate or limit the problem. BTM culture and Somatic Cell Count are cost-effective and easier approach compared to individual quarter milk samples. It seems an appropriate and advisable matrix to work with, approaching MRSA detection and isolation.

A recent paper reported a case of severe LA-MRSA wound infection in a worker of a dairy farm (Soavi et al., 2010). The authors concluded that the work environment could represent a risk factor. Nevertheless, no survey was done in order to determine the real presence of MRSA, and specifically ST398, in that farm. The evaluation of zoonotic potentialities of MRSA and the prevention passes undoubtedly through the determination of this prevalence. There's actually a remarkable gap in this sense and BTM could be the key for mapping the distribution of MRSA-positive farms on a specific geographic area.

The study area of this work is a territory characterized by an heavy presence of livestock of any species and farms of diverse types. The Po valley is a wide and fertile region in a country rich of mountains and where agricultural and industrial activities has found fit sites to settle. Southern Brescia province and similar settings belonging to nearest province constitutes one of the most productive area in Italy. The concentration of dairy farms, pig and poultry holdings of wide size made it become a very rich area but at the same time an epidemiological laboratory where control of diffusive infectious diseases has become a real struggle. The recent outbreaks of Avian flu and Swine Vescicular Disease made all the problems linked to high farming concentration to emerge. Describing a protection zone around an infected herd without including negative herd is extremely difficult if farms are very near. For these reasons a similar area appeared fit to verify the hypothesis that proximity of dairy farms and pig holding could represent a risk for the former to be MRSA-positive.

Global Position System (GPS) and Geographic Information Systems (GIS) has become precious tools for comprehension and management of infectious diseases. Each herd is pointed and localized on a chart through its geographical coordinates. This makes it possible to identify risk factors or obstacle to spreading of pathogens. For instance during the Avian flu outbreaks experienced in recent years, basing on distribution of positive and negative flocks, it was argued

that the A4 Highway worked as a sterilizing strip limiting the circulation of viruses to the areas in the northern side of the street. On the other hand, tracing circular area around an infected farm is easier by computing data than by classical methods and recognizing other herds included in the area is more immediate.

Thus, the present work describes the identification of MRSA-positive and MRSA-negative dairy herds in the area mentioned above, densely colonized and potentially at risk. The identification took place through culture and molecular methods applied to BTM as a matrix. To our knowledge, this is the first work searching for MRSA in BTM by PCR. Where isolates were available, species and *mec*A status were confirmed by PCR and their lineage identity was ascertained by MLST and spa-typing. The localizations of each herd in the regards of pig holding in terms of distance and/or density was the object of statistical analysis in order to determine an odds ratio defining the risk.

MATERIALS AND METHODS

Culture and PCR on Bulk Tank Milk Samples

Dairy farms of the livestock-dense area coinciding with province of Brescia, Mantova and Bergamo were considered. BTM samples were collected from 229 herds resulted positive for *Staphylococcus aureus* in a previous screening. Samples were kept frozen at -20°C up to the analyses. They were thawed at room temperature. An aliquot was incubated overnight before DNA extraction. After a milk sample pre-treatment, in order to remove protein and fat content, the DNA extraction was done following a protocol describe in literature (Cremonesi et al., 2006). PCRs amplifying *Staph. aureus* 23S specific region and *mec*A gene were performed in two independent reactions. One hundred microliters of milk were plated onto Baird Parker agar, in order to confirm *Staph. aureus* positivity. For further confirmation, several colonies per plate were subcultured and tested for coagulase tube test in rabbit plasma. A milliliter was incubated firstly in Mueller Hinton broth added with 6,5 % NaCl and successively in Tryptic Soy broth added with cefoxitin and aztreonam. Both cultural media were incubated for 24 hours at 37°C and 100 µl of the final broth were plated onto MRSA Chromogenic Agar (Pronadisa, Spain). Blue-greenish colonies with halo were considered putatively MRSA and were isolated and subcultured onto Mueller Hinton agar with 6 g/l of oxacillin. Isolates able to grow underwent DNA extraction and a duplex-PCR targeted to *nuc* gene (Baron et al., 2004) and *mec*A (Murakami et al., 1991) gene in order to confirm the results obtained on bulk milk samples.

All BTM samples underwent Somatic Cells Count by an automated somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN).

RS-PCR genotyping

Waiting for MLST and spa-typing results (by now not available yet) and considering the Simpson's Diversity Index observed in part I, we tried to apply RS-PCR to valuate homology between the isolated MRSA. For RS-PCR genotyping, the method rearranged by Fournier et al. (2008), was used. This method is based on the amplification of the 16S-23S rRNA intergenic spacer region. In a total volume of 25 µl, each reaction contained 1x HotStarTaq Master Mix (Qiagen), 800 nM of each primer (G1:GAAGTCGTAACAAGG and L1:CAAGGCATCCACCGT) and ~30 ng of DNA. The PCR protocol was: 95 °C for 15 min, followed by 27 cycles comprising 94 °C for 1 min, followed by a 2 min ramp and annealing at 55°C for 7 min. After a further 2 min ramp, extension was done at 72 °C for 2 min. PCR products were then analyzed using an Agilent 2100 Bioanalyzer with a DNA 7500 LabChip kit (Agilent Technologies, Palo Alto, CA). For interpretation of the results, two patterns were considered different if two or more peaks of the electropherogram differed in size. Grouping of the RS-PCR profiles was obtained with the BioNumerics 5.0 software package (Applied Maths) using the UPGMA (Unweighted Pair Group Method using arithmetic averages) cluster analysis.

Georeferentiation of dairy and pigs farms

All farms in Lombardia region are geocoded and information on their position, number, type and consistency are collected and elaborated by the Epidemiological Regional Observatory (OEVR) of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER). Each herd was localized by Geographic Information System ArcVIEW (ArcGis, 9.3.1, Esri, Redlands, CA) by geographic coordinates. In addition, all finishing pig holdings within the range of 3 Km around each positive and negative dairy farms in the same area were localized. The choice of finishing farms is based on the results of Friese et al. (2012) that found a much higher frequency of MRSA positive dust samples in this type of farm than in breeding ones. Farms rearing pigs for home-consumption were excluded because of their small size and likely low level of epidemiological link with large livestock trading, which is considered an important source of MRSA (EFSA, 2010). The localization of each dairy and pig farm is displayed in figures 9 and 10.

Moreover, the density of pigs population around the dairy farms was estimated in consideration of number of holdings and the declared consistency of each one.

Identification of virulence genes

The DNA was amplified to verify the presence of virulence-associated genes using primers and protocols described in literature. The virulence factors considered were thermonuclease encoded by *nuc* gene, and the enterotoxins encoded by *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel* (Cremonesi et al., 2005); other two enterotoxins gene (*seb*, *see*) were amplified with a different set of primers (Pinto et al., 2005 and Monday et al., 1999). *spa* and *coa* genes, coding for the surface protein A and coagulase respectively, were used to type the isolate on the basis of length polymorphism of the amplimers; *clf*A (clumping factor A), *tsst* (toxic shock syndrome toxin 1), *eta*, *etb* (exfoliatin toxins A and B): all these genes were targeted with the primers published by Akineden et al., 2001. We also searched for leukocidin E *leuk*E (Fournier et al., 2008); LukS-LukF/PV also known as Panton-Valentine Leukocidin, *mec*A (McClure et al., 2006); *sak* (encodes an anti-opsonin and inhibitor of defensins staphylokinase), *fmtB* (a gene encoding a cell-wall-associated protein), *scn* (staphylococcal complement inhibitor), *chp* (chemotaxis inhibitory protein) (Sung et al., 2008); the gene coding for LuKE-LukD and LukM leukocidins (Jarraud et al., 2002); collagen binding protein, *cna* (Zecconi et al., 2006).

Antimicrobial resistance pattern

The MRSA isolates were tested for antimicrobial susceptibility determined by the disk diffusion method, according to CLSI guidelines (2008). The panel included drugs used for mastitis therapy and molecules representative of all antimicrobial classes: amoxicillin/clavulanic acid (AMC, 20/10 μg), ceftiofur (CEF, 30 μg), cefoperazone (CFP, 30 μg), cefquinome (CEQ, 10 μg), penicillin G (P, 6 μg), marbofloxacin (MAR, 5 μg), danofloxacin (DFX, 5 μg), ciprofloxacin (CPR, 5 μg), tetracycline (TE, 30 μg), gentamicin (GM, 10 μg), kanamycin (K, 30 μg), sulphametoxazole/thrimetoprim (STX, 1.25+23.75 μg), vancomycin (VAN, 30 μg).

Statistical analysis

All statistical analyses were computed using SPSS 19.0 for windows (IBM, SPSS Inc., Chicago IL, USA). SCC/ml were transformed in Somatic Cell Score (SCS) to normalize their distribution. Calculation formula used was log2 (SCC/100,000)+3 (Kirk, 1984). Descriptive statistics of SCC

and SCS values were expressed as mean ± SD. Effects of positivity at PCR/culture for both S.aureus and MRSA on SCC and SCS were compared by mean of a non parametric test (U Mann-Whitney) because both SCC and SCS value were not normally distributed (Shapiro-Wilk Test). Different frequencies were compared by mean of a Pearson's Chi-square test. Statistical significance was accepted at $P<0.05$.

The same tests was used to evaluate the effect of number of pig herds and of density of pigs (consistency in pig heads) on the MRSA status of dairy herds, both at PCR and culture. As number of herds and density didn't follow a normal distribution, the non parametric test U Mann-Whitney was applied. The level of acceptance was the same ($P<0.05$).

RESULTS

One hundred-twenty seven out of 229 analyzed BTM samples (55.5%) resulted *S. aureus* positive at culture, whereas in 217 (94.8%) BTM samples the 23S gene specific for *S. aureus* was detected. A total of 53 herds (23.1%) resulted PCR-positive for *mec*A but only in 10 it was possible to isolate effectively MRSA. All frequencies and percentages are summarized in table 4.

Table 4: frequencies and percentages of the results of culture and PCR on BTM samples.

	culture MRSA		**culture Baird Parker**		**PCR mecA**		**PCR 23S (S. aureus)**	
	N°	**%**	**N°**	**%**	**N°**	**%**	**N°**	**%**
Negative	219	95.6	102	44.5	176	76.9	12	5.2
Positive	10	4.4	127	55.5	53	23.1	217	94.8
Total	229	100.0	229	100.0	229	100.0	229	100.0

The status of *mec*A PCR-positive or PCR-negative herd was challenged with the Somatic Cell Count and no significant difference was observed ($P= 0.303$). The difference in SCC and SCS between farms positive and negative at PCR for *S. aureus* (23S gene) was not significant too ($P=0.627$). The fact that BTM resulted *mec*A was not significant to be also 23S-positive ($P=0.073$). The only significant relation was between MRSA culture positive and *mec*A positivity at PCR on total milk ($P=0.001$). Table 5 reports descriptive statistics about the data declared, collected and elaborated by OEVR regarding the presence of finishing pig holding around the dairy herds, irrespective to their MRSA status.

Table 5: number of pig finishing holdings and related total pig heads mean values ± SD included in the 3 km area around each negative and positive (PCR&culture for MRSA) dairy herds.

MRSA culture results		N° pig herds	N° pig heads
Negative	Mean	6.7	8,113
	Standard Deviation	4.1	9,197
Positive	Mean	12.1	18,612
	Standard Deviation	4.6	11,344
Total	Mean	7.0	8,700
	Standard Deviation	4.3	9,601

At least one isolate per herd was typed by RS-PCR, waiting for MLST and *spa* typing. The homology among the isolates is represented in figure 6. Antimicrobial susceptibility testing confirmed resistance to TE and P and sensibility to VAN. Nevertheless, three isolates shown resistance to STX and two out of these were resistant to some extent to aminoglycosides, a feature that isolates of part I didn't display. In isolates 2669 and 2785 resistance to STX and aminoglycosides coexists with resistance to fluoroquinolones that is present in other two isolates.

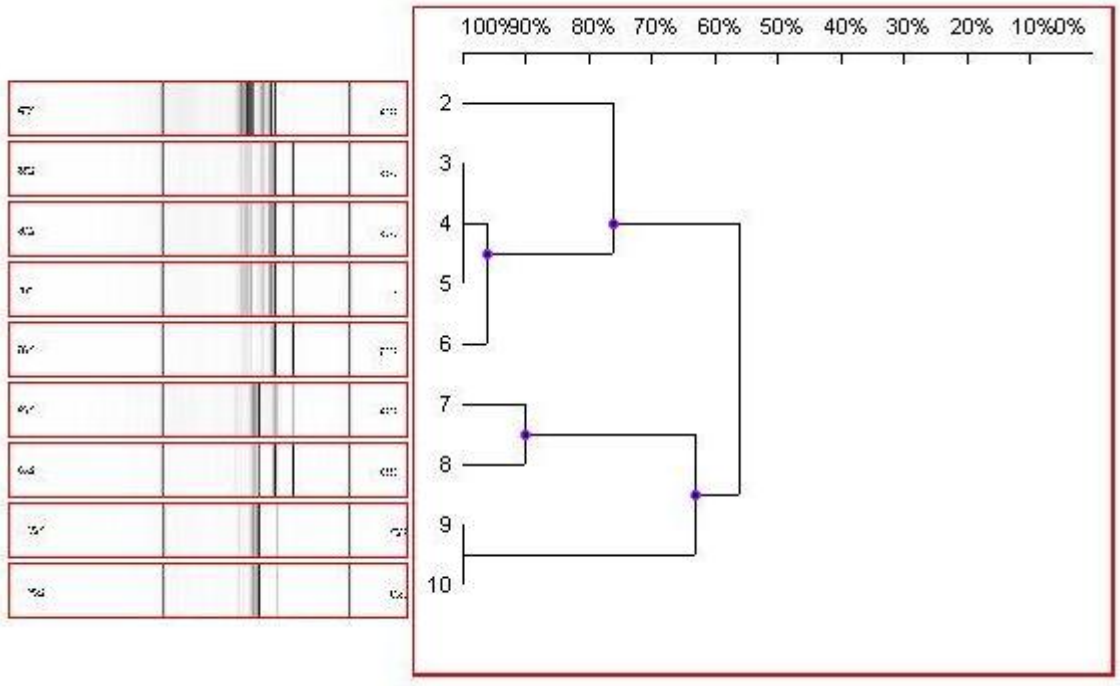

Figura 6: the dendrogram shows graphically the homology (expressed in percentage) among the isolates. Isolates 3 (ID 2785), 4 (2795) and 5 (2601) resulted the same. Isolates 9 (2672) and 10 (2679) constitute another branch, whereas 6 (2605) diverges for only a band. The remaining isolates constitute single distinct divergent branches and are different from any other. The tree is the translation of the banding patterns on the left, resulted from the peaks of the electropherogram.

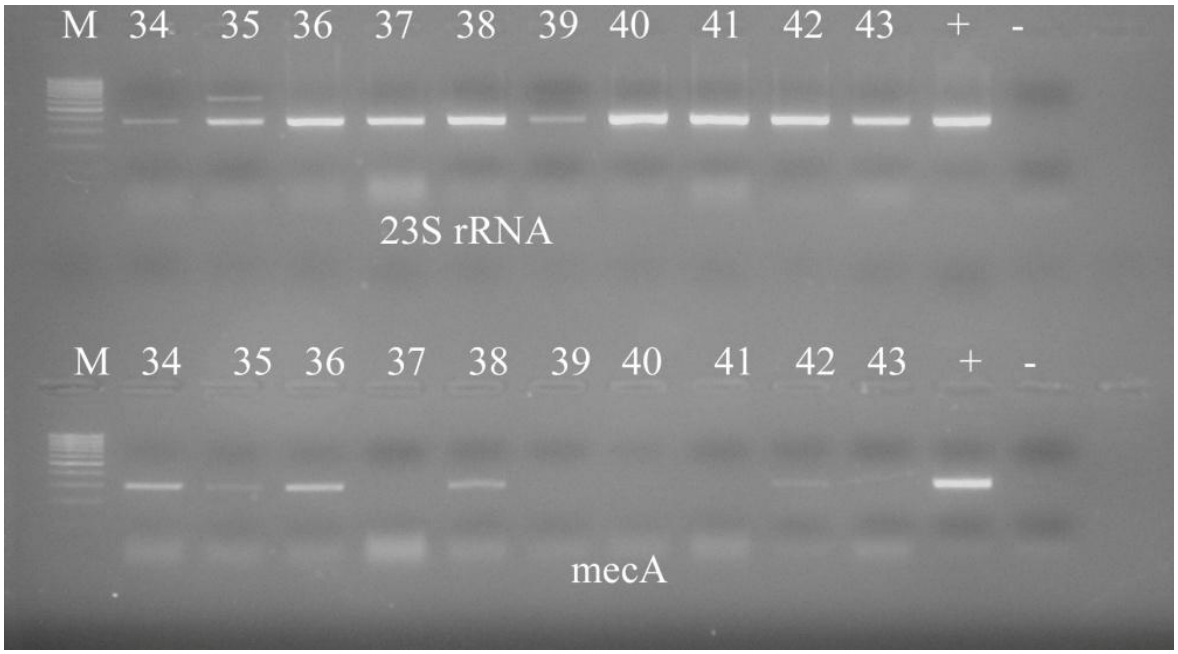

Figure 7: the results of the two distinct PCR reaction performed on total DNA extracted directly from milk. On the top the detection of 23S rRNA for recognition of S. aureus species and below the detection of the *mec*A gene.

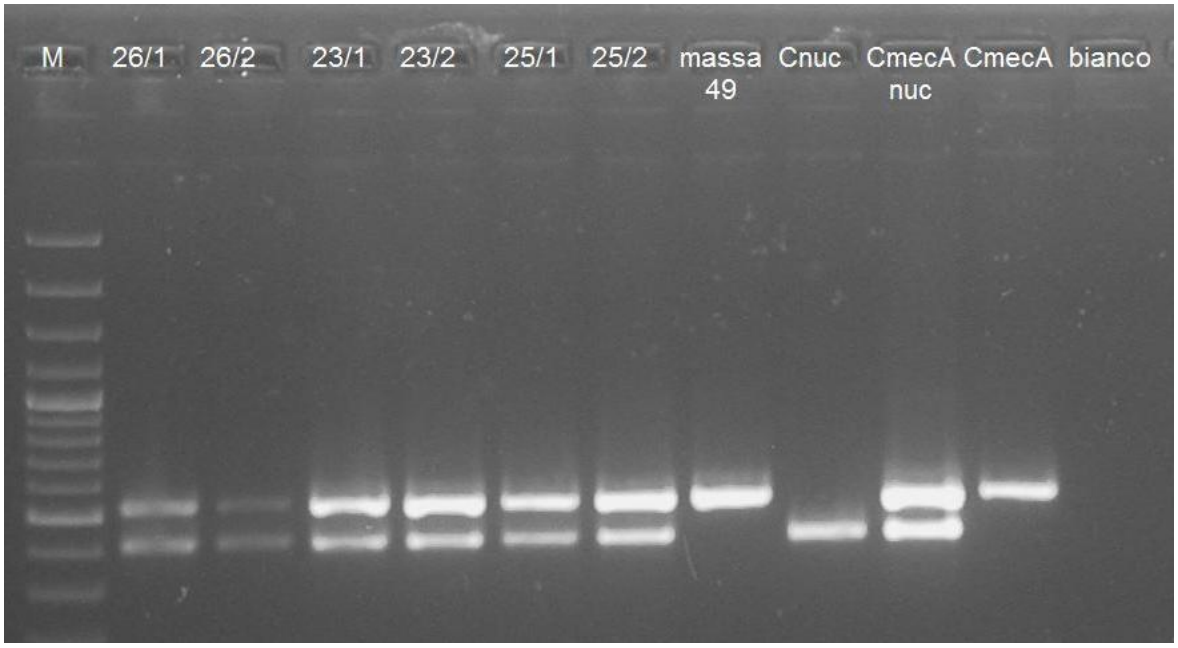

Figure 8: the results of duplex PCR for simultaneous detection in DNA extracted from specific isolates of the *nuc* gene (*S. aureus* species) and *mec*A gene. The isolates from "26/1" to "25/2" are all MRSA whereas isolate "massa 49" is a MRCNS due to the presence of *mec*A gene only.

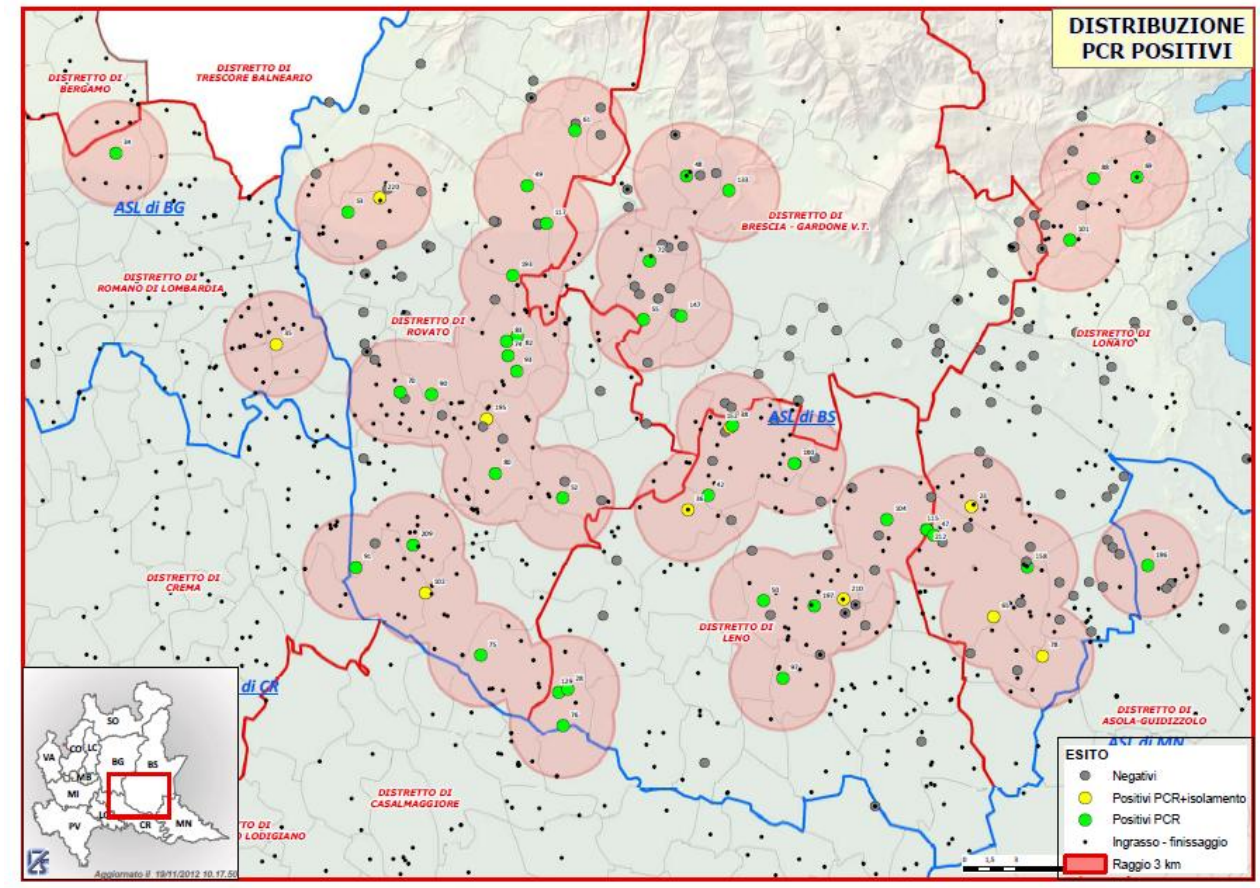

Figure 9: distribution on the study area of the PCR&culture-positive dairy farms (yellow dots), PCR-positive dairy farms (green dots) and negative farms (grey dots). The red halo around farms gives the idea of the 3 km area in which presence of finishing pig farms was considered influencing dairy herds infectious status. The black dots represent finishing pig farms.

The number of finishing pig holdings included in an area of 3 km around dairy herds resulted significantly different comparing negative herds both with PCR-positive herds (P=0.003) and PCR&culture-positive herds (P=0.001). Also the consistencies of pigs population in the area were significantly different comparing negative herds with PCR-positive herds (P=0.005) and PCR&culture-positive herds (P=0.003). It is to be noted the lower P value when only PCR&culture positive herds (with effective isolation of MRSA) are considered.

Figures 9, 10 and 11 represent the territory where the sampled dairy farms and the surrounding pig holdings are spread.

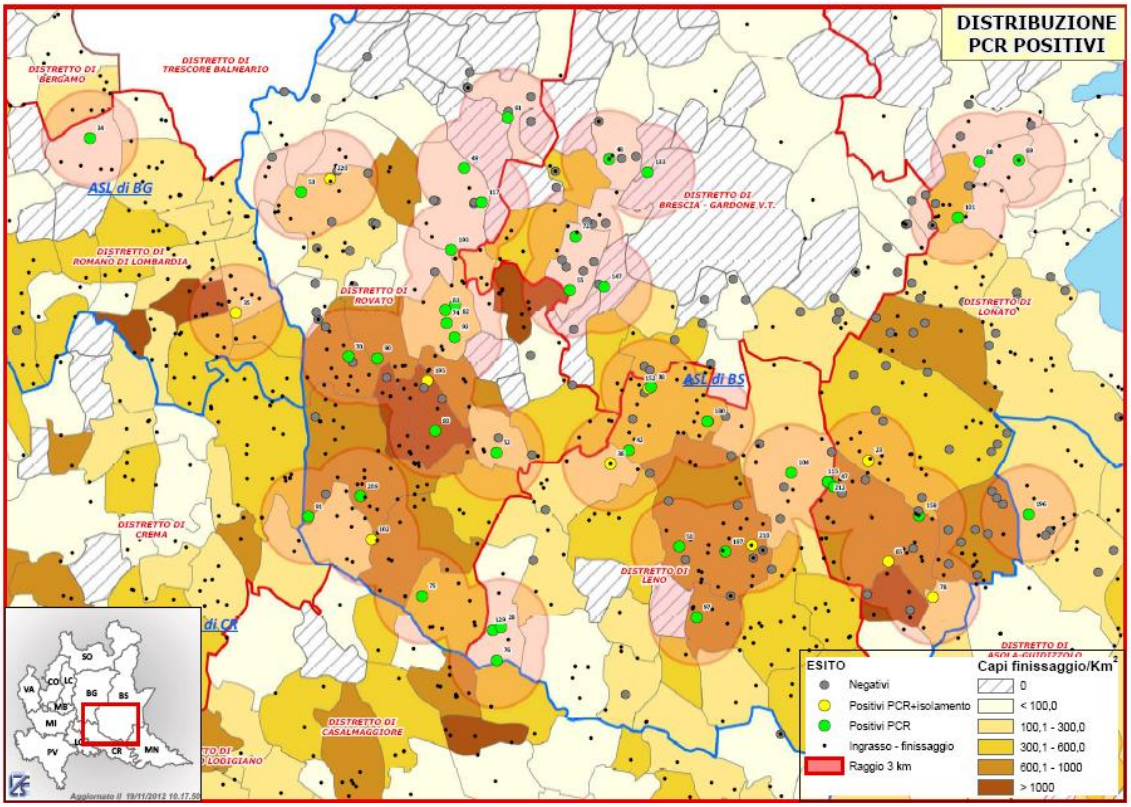

Figure 10: distribution of surveyed dairy farms and scale of density of pigs (total heads including only finishing farms) per municipality.

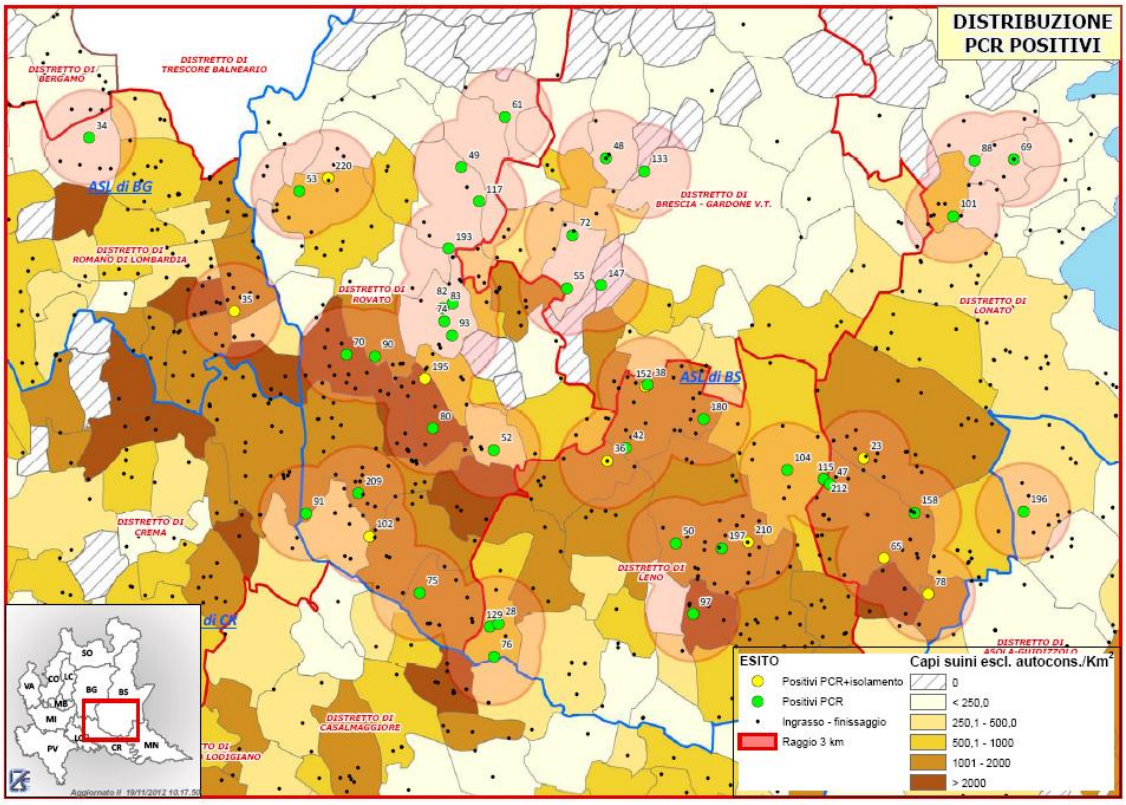

Figure 11: distribution of PCR-positive dairy herds and scale of pigs density including all type of farms (finishing, breeding, etc., excluding only farming for home-consumption) per municipality.

Table 6: results of PCRs for virulence factors analyses and characterization by RS-PCR and *coa* and *spa* genes polymorphism.

Isolate ID	**2759**	**2679**	**2672**	**2601**	**2629**	**2669**	**2785**	**2605**	**2795**
Farm	**152**	**102**	**78**	**35**	**23**	**65**	**195**	**36**	**210**
RS-PCR type		A	A	F	3	3	F	F’	F
Clfa	-	+	+	-	+	+	-	-	-
Coa	>640	>640	>640	>640	>640	>640	>640	>640	>640
leukE	+	+	+	+	+	+	+	+	+
Seb	-	-	-	-	-	-	-	-	-
See	-	-	-	-	-	-	-	-	-
Eta	-	-	-	-	-	-	-	-	-
Etb	-	-	-	-	-	-	-	-	-
Tsst	-	-	-	-	-	-	-	-	-
Spa	>100	>250	>250	>250	>250	>250	>100	>250	>100
Cna	+	+	+	+	+	+	+	+	+
mecA	+	+	+	+	+	+	+	+	+
Nuc	+	+	+	+	+	+	+	+	+
pvl-ED	-	+	+	-	+	+	-	-	-
Chp	-	-	-	-	-	-	-	-	-
Sak	-	-	-	-	-	-	-	-	-
Fmtb	+	-	-	+	-	+	+	+	+
Scn	-	-	-	-	-	-	-	-	-
pvl-M	-	-	-	-	-	-	-	-	-

DISCUSSION

The results confirm the higher sensibility of molecular methods compared to culture both searching for *S. aureus* species and specifically MRSA. To our knowledge, the paper published at present investigated BTM through culture, directly (Virgin et al., 2009) or more often after enrichment (Huber et al., 2010; Haran et al., 2012; Kreausukon et al., 2012), onto some chromogenic agar available in commerce. This is the first survey in which culture has been contemporarily associated to PCR on the total DNA extracted from milk. The findings confirm also the classical issues of comparison between the two approaches. The application of PCR to a matrix allows a better detection of searched target, on the other hand culture allows to characterize and type the effectively isolated bacteria. The limit due to culture lower sensibility reduced the wanted advantage of the concomitant use of both PCR and culture. The lack of MRSA isolation in 43 samples out of 53 could be due non only to sensibility of the medium but also to the fact that the samples were frozen. Anyway, looking to the figures PCR revealed a remarkable prevalence of 23.1%. It is quite higher than those reveled in previous works that at bulk level is usually around 4%. Nevertheless, we can hardly compare these data with those published because of the heterogeneity in sampling, methodologies and media used among the available literature. We started from herds already known as *S. aureus*-positive and most of them confirmed their status. So, this one cannot be considered a formal prevalence study as negative herds were not considered, justifying the high apparent prevalence. In other words, 23.1% should be MRSA prevalence among *S. aureus*-positive farms. However this finding indicates a remarkable diffusion of *mec*A gene-carrying staphylococci in BTM in an area characterized by an heavy potential pressure of pigs as known reservoir of MRSA and MRCNS. Really, the purpose was to define the risk of *S. aureus*-positive farms to be colonized by MRSA or MSSA in relation to the presence of pig holdings. And this hypothesis was demonstrated, founding a significant difference between the number of pigs and pig farms around MRSA- or at least *mec*A-positive dairy herds and those present around absolutely negative dairy herds. Only finishing pig farms were took in consideration as recent papers found this type of farming system as the most likely to be MRSA positive (Broens et al., 2010; Friese et al., 2012). This is probably due to the position of the finishing step at the end of pigs production chain and to the top down diffusion of MRSA from breeding farm throughout the entire chain. Nevertheless, figure 11 suggests that in the study area the finishing farms are the prominent one or at least that composite farms including more than one step of pig production are very common. So, the considerations discussed in this work can be referred to the entire pig population. These findings

should be considered when pig and dairy farms are seated at brief distance (part I) as well as when they coexist in an area characterized by a high concentration of different livestock species in large sized farms. The risk of contamination demonstrated should suggest to screen for MRSA all *S. aureus* positive farms and all isolates from mastitis cases.

Considering by now only MRSA prevalence and the way in which it is determined, recently a nationwide survey in Germany revealed a prevalence of 4.4% on a total of 635 samples (Kreausukon et al., 2012). The starting milk volume was 25 mL in this case, a higher volume than used commonly and in the present work (1 mL). A very low prevalence can be deduced by the survey in the UK which found seven MRSA from five herds among a total of 1,500 BTM (Paterson et al., 2012). In the U.S. Virgin et al. (2009) failed in detection of MRSA in 582 BTM. In that survey only *Staphylococcus* spp. were found *mec*A-positive (i.e. MRCNS); *nuc*-positive and *mec*A-positive duplex PCR were obtained by the mixtures of staphylococcal colonies simulating the mixed staphylococcal flora that can be present in BTM. No individual isolate was however confirmed *nuc*-positive and *mec*A-positive (i.e. MRSA). From a methodological point of view that work was limited by the spreading of milk samples directly onto MRSA chromogenic agar by swabs, without a preventive enrichment. Moreover, the artificial preparation of staphylococcal mixtures presumes a previous selection of staphylococci among those cultured on blood agar and this could have lead to a bias and a likely underestimation of the real BTM samples flora. Whereas, our procedure through extraction of total DNA from pre-incubated BTM guarantees a complete analysis of the wider pool of microorganism really representative of the sample. A more recent survey in the U.S. (Minnesota) found MRSA and matches with the major part of literature that reveals a regional or national prevalence around 4%. The BTM samples were collected in three seasons (spring, summer and fall) from 50 farms which represents only the 2.3% of all the farms enrolled in the State DHIA. The samples were frozen at -20 C° as ours and underwent substantially the same two steps of enrichment, even if on a different selective medium. After isolation, a second selective step was testing the sensibility to oxacillin by MIC, but PCR confirmed only two out of eight suspected MRSA on that basis. This is consistent with the remark of Feßler et al. (2010) about the misleading information given by the sensibility tests towards oxacillin which is not enough to correctly differentiate between *mec*A-positive and *mec*A-negative staphylococci. The detection of *mec*A gene is the gold standard and any phenotypic method has to be followed by a PCR with that target. In Switzerland (Huber et al., 2010) no MRSA was found in BTM (N=100) or raw milk

(N=200) whereas two MRSA were identified from 142 mastitis milk samples (1.2%). Possible explanations for the low MRSA prevalence in Switzerland may be the restrictive and controlled use of antibiotics in farming, a declared good health status of pig herds compared to many countries in the European Union, and the fact that the importation rate of live pigs in Switzerland is very low (<1%). Nevertheless the same survey allowed to identify a prevalence of 62% of MRCNS in BTM. It is remarkable and points out the possibility that some of the *mec*A-positive BTM at PCR on the total DNA in our work could harbor MRCNS instead of MRSA. MRCNS are public and animal health concern as well as MRSA and the horizontal transfer of SCCmec, so their presence within a herd and very likely in udder quarters is a risk for *S. aureus* to become methicillin- resistant through acquisition of this mobile genetic element. The high positivity reported by Huber et al. (2011) in chickens and pigs too seems to confirm that proximity to livestock of other species is a possible source of *mec*A gene. So in the regards of our farms positive at PCR on the templates extracted from total BTM we decided to consider them potentially positive for MRSA.

Neither SCC nor SCS revealed to be influenced by MRSA-status of a herd. Really, the BTM SCC in a herd is conditioned by several factors and the potential presence of MRSA is only one feature linked to the pathogen. Factors such as prevalence of the pathogen, its virulence determinants, other concomitant udder pathogens contribute to SCC as well. In this work 94.8% of herds included were confirmed positive for *S. aureus* at PCR in the bulk. On the basis of the first part of this thesis, it is likely that MRSA or MSSA respectively becomes the dominant, if not the exclusive, *S. aureus* type within a herd. In other words, where MRSA enters a herd it becomes the contagious udder pathogen peculiar to that herd. Anyway, prevalence of *S. aureus* infection within each herd was not determined and the analyses of the virulence factors was made only on MRSA isolates, so a comparison between MRSA and MSSA impact on udder health couldn't be done. The suspected higher pathogenic potential of MRSA compared to MSSA is an issue as well as the different virulence within MRSA strains. CA-MRSA is intended as the strain most prone to cause severe infections in humans, especially in consideration of the frequent association with PVL leukocidin. Spohr et al. (2010) found a significantly higher SCC in udder quarter harboring MRSA than those harboring MSSA. In this work there's no evidence of different behavior of MRSA vs. MSSA based on SCC but it is not possible to exclude that at quarter level some differences could be observed. It could be interesting to evaluate the immunity response, whose SCC is only the most apparent form, in a wide number of udder

quarters affected MRSA and MSSA strains, accompanied by the characterization in the regards of virulence factors and genetic lineages.

Despite absence of an apparent significantly different impact of MRSA on udder health compared to MSSA, presumably in the positive herd MRSA strain must have caused intramammary infections and even mastitis. While pigs are asymptomatic carrier of MRSA and in general *S. aureus* seems to be a commensal microorganism with no effect on animal health, in dairy cows MRSA demonstrated to cause mastitis (Feßler et al. 2010; Spohr et al., 2010; Vanderhaeghen et al., 2010) and the first part of this work confirmed this remark in both the farms A and B. Such ability to cause disease needing a drug treatment is a challenge for a medical approach to farm management. For a resistant and potentially multi-resistant microorganism, to be defined a "bug", it is particularly true. Beta-lactams are the main drugs employed in clinical mastitis treatment and prevention. Fluoroquinolones and tetracycline are administered systemically to sustain host response to infection and to improve cure rate, but MRSA strains are frequently resistant to them. In the present work the occurrence of these resistances was demonstrated as well as the presence of different resistance pattern in relation to the heterogeneity of lineages detected. So at present a farmer can trust on no therapeutic option for MRSA clinical mastitis (Spohr et al., 2010). Culling positive cows after their identification seems to be the only effective policy. Unfortunately, identification of MRSA mammary shedding cows has to face the same troubles that one has to take in consideration for *S. aureus* control too. The fluctuation of *S. aureus* shedding in infected cows could make them undetected at microbial culture and maintain infection within the herd. This is at the origin of well-known *S. aureus* control failure together with poor response to treatment (Zadoks et al., 2011). Moreover, molecular epidemiology demonstrated that more than a strain can be present within a dairy herd and that cow-to-cow transmission cannot explain all infections. Environmental sources have to considered in the complex *S. aureus* epidemiology (Capurro et al., 2010). Searching for MRSA pathway of spreading between pigs and cows and within dairy herds in this thesis, the importance of environment as source and vehicle of MRSA was described. For this reason in case of a MRSA positive herd a complete evaluation of all potential and ascertain reservoir have to be done.

CONCLUSIONS

- Dairy cows are prone to become infected and seems likely acceptor of MRSA more than beef cattle. In Italy there's a lack of wide range prevalence survey, only local or regional ones are available. From those available one can argue that a prevalence of around 4%, in line with international reliefs, can be confirmed. This work agree with this trend. In Italy also misses information about prevalence in meat cattle, so the first statement can seem hazardous. In The Netherlands, a country characterized by high livestock density and high MRSA prevalence, veal calves present higher prevalence even compared to swine. Nevertheless, although such farms are less important in Italy, calves derive from dairies so they should reflect cows prevalence. A study should address this hypothesis. The reason for dairy cows receptivity for MRSA could be due to horizontal transfer during milking by milkmen hands, both as a mean of contamination from cow to cow and as introducer of new strains, coming from the human community (HA-MRSA and CA-MRSA) or from other animals (LA-MRSA).

- Epidemiological picture about MRSA can influence prevalence among and within dairy herds. We demonstrated that relatedness to pig holdings have a significant impact on lactating cows, personnel and the environment. The lineages recovered well represent those recognized in Italian pigs population and demonstrate that ST398 (LA-MRSA) is not the only one involved, even though widely present.

- Common sense, good hygiene and education are key, especially in veterinary practices. Environmental contamination with MRSA acts as a reservoir for infection. Due to high resistance level to most common drugs used for therapy and to zoonotic risk, known MRSA-positive animals should be separated from other animals and possibly culled. Strict hand washing, gloves and gowns wearing and disinfection if in close contact with contaminated environments and matrices are the simplest practices to adopt.

- MRSA can survive in dust for at least several months (Friese et al., 2012). The occurrence of MRSA in dust and consequently in air in pig barns reveals the difficulties to reduce spread of the bacteria within the animal house. A very effective cleaning and disinfection of the stables including all ventilation systems before stocking with new pigs is necessary to avoid transmission of MRSA between subsequent fattening groups or groups of animals within breeding farms by contaminated facilities. Friese et al. (2012) detected MRSA in similar rate in boot swab and in the dust samples, demonstrating that

also inanimate objects could work as passive vector without an adequate disinfection. Airborne MRSA could be one transmission pathway within pig herds and towards neighboring livestock, included dairy cows.

- The study carried out in The Netherlands (Broens et al., 2011) investigating the presence of MRSA in differently structured pig production chains found completely MRSA-negative chains and a strong association between the MRSA status of herds and their suppliers. This suggests that a top-down strategy should be a prerequisite for future control programs which should ensure the absence of MRSA from the entire production chain. The top-down eradication is based on the principle, common to the search-and-destroy policy among humans, of early detection of positive subjects, single patient or herd of animals. Clearly, to the detection of positive animals should follow other strategy than treatment in humans, that should be unrewarding and nonsense. The awareness of positivity of the supplier could suggest to enforce biosecurity and hygienic practices intended to protect animals and humans from contamination. This could be an issue to be considered in farm A and B discussed in the first part of the thesis. The owners should be informed that, beside all the pathogens commonly cause of mastitis, MRSA could enter milking parlor through hands, boots, dust contaminated by the neighbor pig holding if hygiene is not strictly pursued. Using gloves, wearing dedicated boots during milking, preparing accurately each teats, applying an effective post dipping product are common but indispensable practices able to limit the risk.

- Without generating futile fears, MRSA complications in mastitis management and the risks to human health should be known by the farmers. All the workers should be informed that wounds eventually infected by MRSA implies hard therapies. Moreover, attending to the farm duties could made them at least MRSA nasal carrier, with the risk of contamination for household and for health care settings in case of hospitalization. These caveats should bring to special recommendations in working practices such as the use of personal protective equipment, the disinfection of boots to avoid extensive MRSA dissemination, the confinement of dedicated personnel and tools to a specific part of the farm if different species are reared in the same context, deeply washing at least the hands after animal contacts and possibly having a shower before and after each access to the farms, etc.

- Nevertheless, MRSA has been demonstrated a zoonotic pathogen but it could be a so called “humanosis” (Morgan, 2008) as tipically human strains could be transmitted both to production and companion animals. Also the workers involved in at all the levels in animal husbandry should be checked for MRSA carriage, even though it could be only transient rather than permanent. A continuous exposure, like for veterinarians that visit repeatedly positive farms or for farmers that live and work every day in a potentially contaminated environment, coincides with a permanent human contamination.

- Batch treatments with antimicrobials result in a higher MRSA prevalence in pigs herds that were subjected to batch treatments compared herds that were not (Broens et al., 2011). This is an analogy with human health and pet care reliefs, as treatment has always demonstrated to make a patient to be more likely infected by a resistant pathogen. The recommendation to a prudent and controlled use of antibiotics seems to be an essential tool in MRSA confinement.

- The statistically significant role of pig farming density, both in terms of number of herds and number of pigs, confirms the hypothesis that a dairy farm located in proximity to a pig holding is strongly at risk to become infected by MRSA. The risk is stronger, as attested by p value, in the comparison between negative farms and farms whose BTM resulted positive both at PCR and culture. Nevertheless, the risk is significant also in farm whose BTM was positive only at PCR. This could mean that pigs are source above all of the mobile genetic element SCC*mec*, coming both from MRSA and MRCNS. Such element could then shift from a strain to another. The characterization of SCC*mec* will be performed in future and will be useful in confirming this hypothesis.

- Pigs represent a risk for dairy cows and it is advisable to maintain as soon as possible separated the two species. Density around a dairy can have impact due to the air carrying MRSA contaminated around and to the spreading of waste in the fields. Epidemiological links such as personnel and tools movement from farm to farm must not be ignored. Similar issues must be investigated and can be condition by livestock density in an area or can have an independent pathway. Each single context must be surveyed in details.

- The typical LA-MRSA ST398 demonstrated to be able to invade and cause disease in dairy farms. Otherwise other lineages were detected as well, some of them of human origin. This means a complex epidemiology for MRSA and the presence of multiple source of contamination. Much attention should be paid to detect MRSA in milk samples, in particular in mastitis cases. Moreover, typing of the isolates should be performed to understand the likely source of contamination and pronounce prognosis on the basis of known features of specific lineages, especially in case of zoonotic infections.

- The necessity of typing has been confirmed by the heterogeneous behavior (results at MLST and RS, antimicrobial resistance patterns, detected virulence factors) shown by isolates both of part I and II.

- MLST and *spa* typing are surely the best methods to be applied, but they lack of ease of use for the laboratories that work in strict connection with field. Other methods can provide consistent information and can be useful to have an insight in specific situation. Nevertheless, they haven't the same discriminatory power (as demonstrated by Simpson's Diversity Index) and comparability with isolates already typed and universally recosgnized.

- The work demonstrated the advantages of the cooperation between different discipline in studying, preventing and potentially managing infectious diseases: medicine and veterinary medicine, classical culture methods and molecular techniques, statistic and georeferetiation.

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