Review Article

MRSA in Dairy Cattle: A Review

Neeraj Shrivastava[1*] and Arpita Shrivastav[2]

College of Veterinary Science Animal Husbandry, Kuthuliya Rewa, NDVSU, Madhya Pradesh, INDIA

[1]Department of Veterinary Microbiology
[2]Department of Veterinary Pharmacology Toxicology

***Corresponding author:** neerajvet32@gmail.com

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Abstract

Mastitis is a global problem as it adversely affects animal health, affecting quality and the economies of milk production. In the last three decades, world milk production has increased by 50%, from 482 million tons (MT) to 754 million tons in 2012. Apart from mastitis, the chronic shortage of feed coupled with poor quality of fodder is widely regarded as the major constraint in dairy productivity at the production level. Mastitis can occur as per-acute, acute and chronic form which warrants use of antibiotics parenterally as well as locally. Staphylococcus aureus is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of infection. MRSA has emerged as a public health/antimicrobial resistance problem both in human and veterinary medicine. More than 90% of Staphylococcal isolates now produce penicillinase. Resistance to penicillin mainly occurs by spread of resistant strains of MRSA whose clones rapidly spread, often becoming the resident clones and accounting for an increasing percentage of nosocomial infections. Several novel resistance genes have been discovered in MRSA ST398. Due to their characteristic multi-host specificity, ST398 strains can represent an efficient vehicle of the multidrug-resistant determinants that are plasmid-encoded, favoring their transmission and spread.

Key words: LA-MRSA, *mecA* gene, MRSA, MLST, *Staphylococcus aureus*, spa typing

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Introduction

Demand for Milk is Going to Stay

In the last three decades, world milk production has increased by more than 50%, from 482 million tons (MT) in 1982 to 754 million tons in 2012. Since the 1970s, most of the expansion in milk production has been in the South Asia and China (home to 40% of the human population), which is the main driver of milk production growth in the developing world. India is the world's largest milk producer (127.3 MT in 2012) as well as world's largest consumer of dairy products. India produces 16% of global milk production from the 75 million households involved in dairying with low input, low output system. India is still a minor player in the world dairy market with only 1.6% share in global dairy market. Milk production in India is growing at 3.5 % while the consumption is growing at the rate of 5% leaving a gap between demand and supply. To overcome this India needs to double its milk production by 2030. The countries with highest milk deficits are China, Italy, Russian Federation, Mexico and Indonesia. In most of these countries milk is being produced by small holders. The countries with highest milk surpluses are New Zealand, USA, Germany, France, Australia and Ireland. The milk in these countries is produced by intensive dairy farming typically rearing dairy cows at high stocking density (FAO, 2012).

According to the international dairy analyst at Rabobank, medium-term global demand in the global dairy market will increase at a CAGR of above 2% from 2014-2020. This increase will be driven by continuing population growth, urbanization, globalization and increasing disposable incomes leading to rise in per capita milk consumption in the developing countries. For the first time in 30 years, from April 1, 2015 the European Union milk quota for member states have been abolished, allowing the region's farmers to get more active in international markets. It is expected to increase the world's dairy supply of milk (Rabobank, 2015).

Constraints in Milk Production at Production Level - Mastitis

Apart from mastitis, the chronic shortage of feed coupled with poor quality of fodder is widely regarded as the major constraint in dairy productivity at the production level. Mastitis is the most prevalent production disease in dairy herds worldwide and it is well documented as disease with a heavy burden in developed as well as developing countries. It is characterized by physical, chemical and usually, bacteriological changes in milk, and pathological changes in glandular tissues. Mastitis is a global problem as it adversely affects animal health, quality of milk and the economies of milk production, affecting every country including developed ones and causes huge financial losses. There is agreement among authors that mastitis is the most widespread infectious disease in dairy cattle, and from an economic aspect the most damaging (FAO, 2014). Mastitis is a costly disease due to direct losses (a reduction of output and milk quality due to mastitis) and expenditure (additional inputs to reduce the level of the disease) (Hogeveen *et al*., 2011).

Globally mastitis accounts for about 38% of the total direct costs of the common production diseases. In India, the economic losses due to mastitis have increased about 115 folds in the last five decades. The annual economic loss incurred by dairy industry on account of udder infections is estimated to be 7165.51 crores (NAAS, 2013).

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Mastitis and Antibiotics

Antibiotics in livestock enterprise including dairying are used for four reasons viz., therapeutic, prophylaxis, metaphylaxis and growth promoter. More antibiotics are consumed in the livestock enterprise including poultry than in human medicine (Landers *et al*., 2012). Mastitis remains to be single major reason to use antibiotics in veterinary practice. Historically when lyophilized penicillin preparations were made available for the first time to the veterinarians, it was used for the treatment of bovine mastitis as intra-mammary infusions (Gustafson and Bowen, 1997). Mastitis can occur as per-acute, acute and chronic form which warrants use of antibiotics parenterally as well as locally. In addition, mastitis in dairy cattle occurs as clinically and sub-clinically and is treated both in lactating and dry cows (Radostits *et al*., 2000).

***Staphylococcus aureus* is a Major Mastitis Pathogen**

Staphylococcus aureus is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of infection and its capacity to adapt to different environmental conditions. Most mastitis is of bacterial origin, with just a few species of bacteria accounting for most cases. Mastitis pathogens are categorized as contagious and environmental. Contagious pathogens live and multiply on and in the cows' mammary gland and are spread from cow to cow, primarily during milking. Contagious pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, Mycoplasma spp. and *Corynebacterium bovis* (Radostits *et al*., 2000)

Many countries from Asian countries have reported that *Staphylococcus aureus* is the chief etiological agent of mastitis in cattle and buffaloes (NAAS, 2013). *Staphylococcus aureus* is a well-known commensal and pathogen of a large number of animal species, including humans. A wide variety of infections can be caused by *Staphylococcus aureus*, from superficial skin and soft tissue infections to life-threatening septicemia. *Staphylococcus aureus* represents a serious public health burden in both hospital and community settings as well as an economic and animal welfare problem in dairy farming.

Although antibiotic treatment is an option for individual animals, it is unfavorable because of costs and the potential risk of development of antibiotic resistance, and is unsuitable for addressing the problem of long-term persistence of pathogenic *Staphylococcus aureus* in udder tissue (Fluit, 2012).

***Staphylococcus aureus* is a Clonal Bacteria**

It was known as early as 1935 that the *Staphylococcus aureus* isolated from various animal species differ from each other. At that time characterization of *Staphylococcus aureus* isolates by means of phenotypical traits (phage typing, hemolysin types, coagulase types, staphylokinase etc.) suggested the existence of host-adapted so-called ecovars such as for humans, cattle, sheep, and chicken (Devriese, 1984). Use of different methods of DNA-based molecular typing (e.g. macro restriction patterns, PCR-based typing) revealed that among *Staphylococcus aureus* from different mammalian host species clones with distinctive genetic

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background are responsible for the majority of infections within each host species, although distinct ''bovine types'' are often interspersed among the human genotype clusters suggesting that human-adapted MRSA were the evolutionary ancestors of *Staphylococcus aureus* adapted to cattle in modern agriculture (Zadoks *et al*., 2000).

Staphylococcus aureus Acquires Antibiotic Resistance Quickly

The mortality of patients with *Staphylococcus aureus* bacteremia in the pre-antibiotic era exceeded 80%, and over 70% developed metastatic infections. After penicillin came into existence in 1940 Staphylococcal infection showed better prognosis and in 1942 penicillin-resistant staphylococci were recognized. Within 20 years more than 80% of both community and hospital-acquired staphylococcal isolates were resistant to penicillin. More than 90% of staphylococcal isolates now produce penicillinase, regardless of the clinical setting. The gene for β-lactamase a transposable element found on a large plasmid, with additional antimicrobial resistance genes (e.g., gentamicin and erythromycin). Resistance to penicillin mainly occurs by spread of resistant strains.

In 1959 methicillin, the semisynthetic penicillinase resistant penicillin came into existence hand in hand followed by entry of methicillin-resistant isolates. Introduced in British hospital MRSA clones rapidly spread across international borders. Waves of clonal dissemination with different dominant phage types were reported. They were found to be responsible for a large proportion of cases. Once identified in a new setting, these unique MRSA clones rapidly spread, often becoming the resident clones and accounting for an increasing percentage of nosocomial infections. Like the penicillin resistant strains, the MRSA isolates also frequently carried resistance genes to other antimicrobial agents. (Franklin and Lowy, 2003)

Table 1: Antibiotic discovery and resistance development in *Staphylococcus aureus*

S. No.	Antibiotics	Discovered	Introduced into Clinical Use	Resistance Identified	% Resistant Animal Isolates
1	Penicillin	1928	1943	1940	100%
2	Methicillin	1959	1959	1961	13-34 %

(EMEA, 1997)

Resistance to antistaphylococcal beta-lactamase stable penicillins like methicillin has been historically referred to as methicillin-resistant *Staphylococcus aureus* or MRSA. The acronym MRSA is still commonly used even though methicillin is no longer used for treatment. Antimicrobials like oxacillin and nafcillin now are used for treatment of *Staphylococcus aureus* infections. The acronym ORSA (oxacillin-resistant *Staphylococcus aureus*) may be used interchangeably with MRSA. The first communication on MRSA infections in domestic animals concerned mastitis cases in dairy cows in Belgium in 1972 (Devriese *et al*., 1972). Since that time, there have been reports of sporadic cases of infections with MRSA in a variety of other domestic animal species such as horses, chickens, dogs and cats (Weese, 2010)

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Bottleneck and Founder Effect Promotes Spread of Resistance

Genetic bottlenecks are defined as "events in which the number of individuals in a population is reduced drastically." Population Bottleneck: is event in which a large number of a individual are killed causing the remaining population to reproduce. This causes all of the offspring to have similar traits as the ones remaining after the others were killed. As with *Staphylococcus aureus*, a few bacteria were resistant to penicillin. Therefore, they were selected over and over again to reproduce, until the entire population of bacteria became resistant to penicillin. A new antibiotic called methicillin became available in 1959 to treat penicillin-resistant bacteria strains, but by 1997, 40% of hospital staphylococci infections were caused by methicillin-resistant *Staphylococcus aureus*, or MRSA. Now, community-acquired MRSA (CA-MRSA) can spread freely through the general populace, particularly when people are in close contact. More recently livestock – associated MRSA has been reported to spread among the livestock and humans (Mader and Windelspecht, 2013).

Types of MRSA – Based on Putative Source of Infection

Human MRSA infections have been categorized into three groups based on their putative sources: health care-associated MRSA, community-associated MRSA and health care-associated MRSA with community onset. A fourth category has recently been added to describe human MRSA cases associated with exposure to livestock (livestock-associated MRSA [LA-MRSA]). Human colonization with LA-MRSA multilocus sequence type 398 (ST398) was first recognized among swine farmers in France and The Netherlands in the early 2000s. Since those early reports, ST398 and closely related STs within CC398 have been reported in diverse livestock hosts in many countries around the world. Human cases of MRSA CC398 have also been increasing rapidly and now account for up to 25% of the total MRSA cases in some parts of The Netherlands. Given its rapid emergence and trajectory of increasing importance in humans, the evolutionary history of MRSA CC398 has relevance for the epidemiology of MRSA and global health.

Table 2: Types of Methicillin-Resistant *Staphylococcus aureus* (MRSA) based on putative source of infection

S. No.	Types of MRSA	Year	Host Specificity
1	Health care-Associated – MRSA (HA-MRSA)	1961	Humans
2	Community Associated – MRSA (CA-MRSA)	1981	Humans
3	Health care-Associated – MRSA with Community Onset - Invasive (HACO- MRSA)	Late 1990s	Humans
4	Livestock Associated – MRSA (LA-MRSA)	2005	Pigs, Cattle, Poultry, Humans

Mechanism of Methicillin Resistance in LA- MRSA

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Resistance Due to *mecA* Gene

Methicillin resistance requires the presence of the chromosomally localized *mecA* gene. *mecA* is responsible for synthesis of penicillin-binding protein 2a (PBP2a; also called PBP2′) a 78-kDa protein. PBPs are membrane-bound enzymes that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains. Their activity is similar to that of serine proteases, from which they appear to have evolved. PBP2a substitutes for the other PBPs and, because of its low affinity for all β-lactam antibiotics, enables staphylococci to survive exposure to high concentrations of these agents. Thus, resistance to methicillin confers resistance to all β-lactam agents, including cephalosporins. Recent studies determined the crystal structure of a soluble derivative of PBP2a. PBP2a differs from other PBPs in that its active site blocks binding of all β-lactams but allows the transpeptidation reaction to proceed. The *mecA* gene is located on a mobile genetic element, named staphylococcal cassette chromosome *mec* (SCC*mec*) inserted in the *Staphylococcus aureus* chromosome upstream *orf* X (Katayama *et al.*, 2000).

(a) Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin. I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR1-blaI*. In the absence of penicillin, β-lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI* to commence. V–VII. β-Lactamase, the extracellular enzyme encoded by *blaZ* (V), hydrolyzes the β-lactam ring of penicillin (VI), thereby rendering it inactive (VII).

(**b**) Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. Exposure of MecR1 to a β-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and β-lactamase.

Resistance Due to *mecC* Gene

A recently published article (Garcia-Alvarez *et al*., 2011) reported the presence of MRSA strains with unusual features in bovine milk samples from the UK. These strains belonged mainly to CC130 (more rarely to CC425) and carried a novel *mecA* gene that was only 70% identical at the nucleotide level to the classical *mecA* gene, escaping detection by routine PCR assays. Similarly, the encoded PBP2a protein was only 63% identical to the classical PBP2a protein and could not be detected by the commercially available antibody-based assays. The novel *mecA* was named *mec*ALGA251, after the designation of the bovine strain where it was identified for the first time. In turn *mec*ALGA251 was part of a novel SCC*mec* element,

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identified as type XI. Other distinguished features of the SCC*mec* type XI are the presence of a *mec* complex that has a different organization from other SCC*mec* elements including also the beta-lactamase resistance gene *blaZ* and the presence of an arsenic resistance operon.

Although previously CC130 had been associated only with methicillin-susceptible *Staphylococcus aureus* from animals, isolates with the same novel *mecA*LGA251 were recovered from human clinical infections in the UK and Denmark. The ability of these strains to cause infections in humans was confirmed by a concurrent report describing two patients infected with similar strains in Ireland and by another study from Germany where 11 CC130 strains carrying SCC*mec* typeXI and *mecA*LGA251 were identified out of a collection of 12,691 MRSA obtained in 2006–2011 (Cuny *et al.*, 2011).

Laboratory Identification of LA-MRSA

Accurate and rapid identification of methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical specimens in the laboratory is essential for timely decisions on isolation procedures and effective antimicrobial chemotherapy. As LA-MRSA has got great public health importance, it is also required for monitoring of prevalence, genetic diversity / molecular typing and multi-drug antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) (EFSA, 2012). So essentially it is a two-step procedure which comprises of –

1. Identification of the Methicillin resistance.
2. Identification of the clone by the molecular typing methods.

Accurate detection of MRSA can be difficult because susceptible and resistant populations may coexist in the same culture. This heteroresistance is a problem in the clinical laboratory because the resistant population may grow more slowly than the susceptible population. Depending upon the available facility and objective of investigation both phenotypic and genotypic methods are used for the detection of MRSA.

Phenotypic Methods (CLSI, 2013)

a) Broth Dilution Method

The CLSI method requires the use of MH broth, an inoculum of 5 · 10^5 cfu/mL and incubation at 33–35ºC for 24 h, is the only defined method in general use.

Interpretive Criteria (in µg/ml) for Cefoxitin MIC Tests			
	Susceptible	**Intermediate**	**Resistant**
Staph. Aureus	≤ 4 µg/ml	N/A	≥ 8 µg/ml
CoNS	N/A	N/A	N/A

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b) Disc Diffusion Method

Disk diffusion is one of the oldest approaches to antimicrobial susceptibility testing (AST) and remains one of the most widely used AST methods in routine clinical laboratories.

Medium: Mueller-Hinton agar

Inoculum: McFarland 0.5

Incubation: Air, 35±1ºC, 18±2h

Reading: Read zone edges as the point showing no growth viewed from the back of the plate against a dark background illuminated with reflected light.

Quality control: *Staphylococcus aureus* ATCC 29213

This method is based on Kirby Bauer disc diffusion assay. It uses Mueller Hinton agar and cefoxitin disc (30 µg).

Interpretive Criteria (in mm) for Cefoxitin Disk Diffusion Test			
	Susceptible	**Intermediate**	**Resistant**
S. aureus	≥ 22 mm	N/A	≤ 21 mm
CoNS	≥ 25 mm	N/A	≤ 24 mm

Fig. 1: Disk diffusion assay using Cefoxitin disc

c) E test

Etest® is a well-established method for antimicrobial resistance testing in microbiology laboratories around the world. Etest® consists of a predefined gradient of antibiotic concentrations on a plastic strip and is used to determine the Minimum Inhibitory Concentration (MIC) of cefoxitin.

Interpretive Criteria (in µg/ml) for Cefoxitin Etest Strips			
	Susceptible	**Intermediate**	**Resistant**
S. aureus	≤ 6 µg/ml	N/A	≥ 6 µg/ml
CoNS	N/A	N/A	N/A

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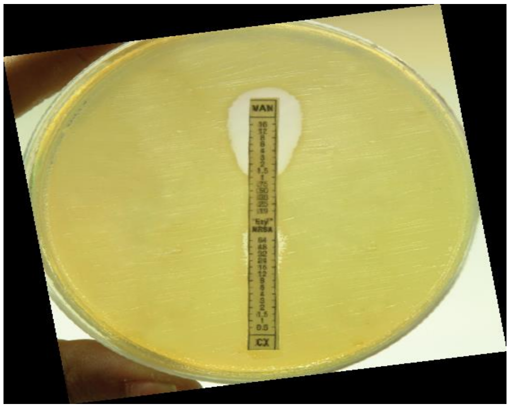

Fig. 2: E test

d) Latex Agglutination Test

A rapid (10 min for a single test) slide latex agglutination test based on detection of PBP2a is commercially available as a kit from several suppliers. The method involves extraction of PBP2a from suspensions of colonies and detection by agglutination with latex particles coated with monoclonal antibodies to PBP2a. The test is very sensitive and specific with S. aureus. The method requires no special equipment and is suitable for confirmation of resistance or equivocal tests in routine clinical laboratories. Isolates producing small amounts of PBP2a may give weak agglutination reactions or agglutinate slowly. Reactions tend to be stronger if PBP2a production is induced by growth in the presence of penicillin. Rare isolates may give negative reactions.

Fig. 3: Latex Agglutination test for the detection of PBP2a

1. Genotypic Methods

a) Identification of *mec*A gene by Polymerase Chain Reaction (PCR)

Molecular testing needs to be performed on the presumptive positive MRSA isolates to confirm methicillin resistance (gold standard). In this view, it is necessary to perform *mecA* gene detection using simple or multiplex PCR amplification methods. It is recommended that a reliable PCR method be used and that the

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procedures be harmonized (EFSA, 2012). For this purpose, it is recommended that a multiplex PCR be used that allows, in the same PCR:

i. species identification of *Staphylococcus aureus* with detection of the staphylococcal protein A gene (*spa*);
ii. detection of the methicillin resistance determinant, including both *mecA* and the recently described *mecC*; and
iii. simultaneous detection of the Panton Valentine Leukocidin (pvl) gene.

Typing Methods for Identification of LA- MRSA Clone

A thorough knowledge of the dissemination and the molecular epidemiology of MRSA strains are required to develop effective strategies to prevent the spread of MRSA. The existence of two major host-associated *Staphylococcus aureus* CC398 clades emphasizes the need for rapid molecular genotyping methods in epidemiological investigations and source tracking of *Staphylococcus aureus* CC398. Various molecular typing techniques have been developed to investigate the spread and evolution of MRSA The most commonly used techniques include-

1. Pulsed-Field Gel Electrophoresis (PFGE),
2. Multi Locus Sequence Typing (MLST),
3. Staphylococcal Protein A (spa) typing, and
4. Staphylococcal Cassette Chromosome (SCC) mec typing.

1. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is still considered to be the golden standard for typing of MRSA isolates, and is one of the most discriminative typing methods. Therefore, PFGE is used to study MRSA origins and transmission. PFGE is based on the digestion of chromosomal DNA with the restriction enzyme *SmaI*, followed by agarose gel electrophoresis. The PFGE patterns are analysed with a software package with Dice comparison and unweighted pair group matching analysis (UPGMA) settings according to the criteria of Tenover et al. Attempts to harmonize PFGE protocols and to establish a standardized nomenclature have proven only partially successful when judged by reproducibility, speed, and costs of analysis. Because of the need for strict adherence to standardized protocols, common databases were only realised on a national level, such as in the USA and The Netherlands. However, on an international level, attempts for a common PFGE nomenclature were not successful.

2. Multilocus Sequence Typing MLST

Multilocus sequence typing (MLST) is an unambiguous procedure for characterising isolates of bacterial species using the sequences of internal fragments of (usually) seven house-keeping genes. Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci

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define the allelic profile or sequence type (ST). Each isolate of a species is therefore unambiguously characterized by a series of seven integers which correspond to the alleles at the seven house-keeping loci. In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The rationale is that a single genetic event resulting in a new allele can occur by a point mutation (altering only a single nucleotide site), or by a recombinational replacement (that will often change multiple sites) - weighting according to the number of nucleotide differences between alleles would erroneously consider the allele to be more different than by treating the nucleotide changes as a single genetic event. MLST has been developed for *Staphylococcus aureus* by Mark Enright (now situated at the University of Bath) in the laboratory of Professor Brian Spratt, Imperial College, London, in collaboration with the laboratories of Drs Nick Day and Sharon Peacock at the John Radcliffe Hospital, Oxford (Enright *et al*., 2000).

Obtaining an Allelic Profile and Comparing the Strains with Those in the Database

The primers for the amplification and sequencing of these gene fragments can be obtained www.mlst.net. The sequences must be obtained on both strands, and they must be 100% accurate, since even a single error may convert a known allele into a novel allele. The result is an allelic profile or Sequence Type (ST). The CC 398 clone, for example, has MLST profile 3-35-19-2-20-26-39, which has been defined as ST398 (www.mlst.net). The MRSA nomenclature is currently based on MLST and the SCCmec type. For example, the LA- MRSA CC398 clone is ST398 -MRSA-IV, harbouring SCCmec type IV. The software package BURST (Based Upon Related Sequence Types) is used to define clonal complexes (CCs) and to investigate evolutionary events within the MRSA population (www.mlst.net). When 5 of the 7 housekeeping genes have identical sequences, MRSA isolates are grouped within a single CC. The ST with the largest number of single locus variants (SLV) is the ancestor of a CC. Furthermore, subgroup founders can be described as SLVs or double locus variants (DLV) of a founder of a CC that has become prevalent in a population, and may be diversified to produce its own set of SLVs and DLVs. A disadvantage of MLST is that it is rather expensive, laborious and time consuming. (www.mlst.net)

3. Staphylococcal Protein A (*spa*) typing

The sequences of the polymorphic region X of the *Staphylococcus aureus* protein A (spa) gene have been used by Frenay *et al*. to developed a single-locus sequence typing technique for MRSA. The spa locus consists of a number of mainly 24-bp repeats, and its diversity is attributed to deletions and duplications of the repeats, and, more seldom, to point mutations. The discriminative power of spa typing lies between that of PFGE and MLST, and, in contrast to MLST, spa typing can be used to investigate both the molecular evolution and hospital outbreaks of MRSA. The main advantage of spa typing over MLST is its simplicity, since it involves sequencing of only a single locus. Another advantage of spa typing is that several

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laboratories can use different sequencing platforms and analyze the resulting sequence chromatograms using special software. By this means, typing is made accessible not only to reference laboratories, but also to local laboratories. Comparability and a common nomenclature with excellent quality of data are available. For infection control purposes, it is possible to collect spa typing data continuously, and it can easily be adapted for electronic early warning algorithms for the automatic detection of MRSA outbreaks in regions or institutions where MRSA is endemic with a pool of heterogenic circulating spa types. Because of its higher discriminatory power, several spa types correspond to a single ST as determined with MLST, but they remain within an assigned clonal cluster. The implementation of the clustering algorithm Based Upon Repeat Patterns (BURP) into StaphType makes cluster analysis based on spa typing data (spa clonal complexes) possible. A recent study has shown a very good concordance between PFGE, MLST and spa typing using BURP analyses.

Harmsen *et al.* (2003) used a novel software for spa typing that met the requirements of modern, internet-based management of genotyping data. The Ridom StaphType software (Ridom GmbH, Würzburg, Germany) is most widely used for the analysis of spa sequence in Europe (EFSA, 2012). The laboratory typing data are synchronized with the central spa server (www.spaserver.ridom.de), which is curated by the SeqNet.org initiative (www.seqnet.org), ensuring a universal nomenclature and public access to the typing data. Currently the spa server databases is one of the largest known sequence-based typing of databases of *Staphylococcus aureus*, and comprises over 14172 types consisting of a combination of 654 spa repeats from nearly 310331 isolates typed in 110 countries worldwide (www.spaserver.ridom.de).

4. Staphylococcal Cassette Chromosome mec (SCCmec) typing

Mobile genetic element SCC mec carrying the mecA gene is responsible for Methicillin resistance on the organism. On the basis of nucleotide differences, the SCC mec elements are divided into two essential components, ccr (cassette chromosome recombinase) gene complex, represented by ccr genes and mec gene complexes. Eight major types of SCC mec elements were reported till recently but three more new types have been added recently from bovine and human origins altogether eleven SCCmec types. HA-MRSA isolates contain mainly type I, II, and III SCCmec elements while CA-MRSA contain type IV and V. Majority of Indian HA-MRSA collected between 2002 and 2006 contained type III or IIIA SCCmec elements. To date, the website of the International Working Group on the *Staphylococcal* Cassette Chromosome elements lists 11 types of SCC*mec* elements, originating from the combination of eight different *ccr* genes complex and five different *mec* gene complex.

Livestock Associated - Methicillin Resistant *Staphylococcus aureus* in Dairy Cattle

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Host Range

LA-MRSA ST398 has been found to be associated with pigs, veal calves, dairy cattle, turkeys, broiler chickens, companion animals, black rats and people in close contact with livestock (Vanderhaeghen *et al*., 2010). Pigs and cattle have been shown as reservoir of LA- MRSA. Like humans a study in the Switzerland has shown that LA-MRSA CC 398 can colonize the nostrils of the cattle. However, in dairy cattle the udder seems to be the major source of infection and reservoir.

Transmission

LA- MRSA spreads by direct contact. It has been also reported to be present in the environment and skin but its role in transmission has not been studied. The spread of MRSA ST398 among livestock throughout the world within a few years is not understood. In general, it is well accepted that antibiotic use selects for resistant organisms, but this explanation is too simple. Also, no worldwide change in antibiotic policies appears to have occurred. Therefore, it is unlikely that antibiotic usage alone can explain the spread.

Virulence Factors

Successful infection of both humans and animals depends on virulence factors produced by *Staphylococcus aureus*. A wide spectrum of secreted and cell surface-associated virulence factors can be expressed to promote adhesion to the host extracellular matrix components, damage host cells, and fight the immune system. At least 25 different toxins, 15 microbial surface components recognizing adhesive matrix molecules, which are important for adhesion to tissues, 20 immune evasion molecules and several other virulence factors are known.

The majority of these virulence factors have been identified in isolates of human origin, and only a few studies have investigated virulence genes in non-ST398 *Staphylococcus aureus* from chickens and cows. However, some novel virulence factors have recently been identified in mastitis and livestock-associated *Staphylococcus aureus*. Smyth *et al.* investigated the presence of the superantigen genes sae–see, seg–seo, and seq, as well as the toxic shock syndrome toxin 1 gene, in isolates from a number of animals, including 15 chicken and 99 cow isolates. Among cow isolates, no particular gene was dominant, but sea, seb, see, seh, sek, and seq were lacking in this collection. In one study a larger collection of virulence genes in 76 *Staphylococcus aureus* isolates from clinical cases of bovine mastitis from all over The Netherlands showed variation in the presence of the genes encoding the different superantigens. In addition, the presence of the genes for a number of additional virulence factors, including adhesins, proteases, and capsule type, was investigated. For the adhesins, the genes for fibronectin-binding protein A, elastin-binding protein and extracellular fibrinogen-binding protein were almost always present, as was the gene for capsule type 8 (96%). Only one isolate encoded staphylococcal complement inhibitor, chemotaxis inhibitory protein of *Staphylococcus aureus*, and staphylokinase. This suggests that the isolate may have a human origin, because

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these virulence factors show activity only against the human innate immune system. The sec3/sel/tst signature of the bovine staphylococcal pathogenicity island (SaPIbov) was present in only 20% of the isolates; 10% of the isolates lacked one of the genes, and thus appeared to contain an incomplete or variant SaPIbov. The isolates could be clustered into six major groups on the basis of their virulence gene content. This clustering agreed with typing performed with multi-locus sequence typing and pulsed-field gel electrophoresis (Fluit, 2012). Panton–Valentine leukocidin (PVL) has been reported in several Chinese ST398 isolates. This toxin is common among community-associated MRSA strains. For example, the dominant community-associated MRSA strains in the USA and Europe, USA300 and ST80, encode PVL, and it has been suggested that PVL contributes to the wide dissemination of these strains.

Multi-Drug Resistance in LA-MRSA

ST398 isolates appear to be universally resistant to tetracyclines, owing to the presence of the tet(M) gene on a chromosomally located transposon, often in combination with the plasmid-encoded tet(K) gene. However, high rates of resistance to other antibiotics have been reported for ST398 from pigs. For example, a Belgian study showed that, among 643 MRSA isolates, 97% were resistant to trimethoprim, 73% to lincosamides, and 32% to fluoroquinolones. However, rates may vary according to several factors, including country, type of farm and age of the animals. Several novel resistance genes have been discovered in MRSA ST398. These include: the apramycin resistance gene ampA, vga(C), which encodes resistance to streptogramin A, pleuromutilin, and lincosamides, dfrK, which encodes resistance to trimethoprim, and vga(E), which encodes resistance to streptogramin A, pleuromutilin, and lincosamides. The last of these genes is integrated in the well-known transposon Tn554. The dfrK gene was also seen as part of a new combination of plasmid-borne resistance genes that also included erm(T), which encodes macrolide–lincosamide–streptogramin A resistance, and tet(L), which encodes resistance to tetracycline. It is likely that, sooner or later, these genes will also be transferred to human-specific lineages, thereby further compromising our ability to treat *Staphylococcus aureus* infections.

LA-MRSA ST398 is generally susceptible to antibiotics other than beta-lactams, but it is characteristically resistant to tetracycline, which suggests that heavy tetracycline use in the pig industry may have favored the emergence of this clone.

Phylogenetic Clade

The livestock-associated *Staphylococcus aureus* CC398 clade evolved from the basal human clade, and that this human-to-livestock host jump was accompanied by the loss of a bacteriophage (ΦSa3) harboring *scn* and functionally related genes that encode modulators of human innate immunity (IEC) and acquisition of a Tn916-like transposon carrying the tet(M) gene that confers resistance to tetracycline, which is commonly used in livestock production. (Stegger *et al*., 2013).

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Public Health Significance

Staphylococcus aureus CC398 has been identified in humans with no apparent livestock-associated risk factors in several geographically diverse areas, including the People's Republic of China, Denmark, France, French Guiana, the Caribbean, and the United States. Subsequently, whole-genome sequence analysis of *Staphylococcus aureus* CC398 isolates from these geographic areas demonstrated that they belong to the human clade. By use of the assays reported here, we identified the first cases of *Staphylococcus aureus* CC398 belonging to the human clade in Algeria, Belgium, Finland, India, and the Netherlands. The majority of these cases had no prior exposure to livestock. These results underscore the usefulness of integrating these assays into *Staphylococcus aureus* CC398 surveillance programs and epidemiological studies.

In Belgium 37.8% of pig farmers were found colonized by ST398; in Germany 86% of pig farmers and 45% of veterinarians caring for pigs were colonized. Family members that were not directly exposed to pigs were colonized in a lower percentage (Cuny *et al.*, 2010), indicating that inter-human transmission can occur but at low frequency. Veterinarians and other attendees of an international conference on pig health in Denmark were sampled for MRSA carriage and 12.5% were found to carry MRSA that mostly belonged to *spa* types corresponding to ST398. Infection can follow colonization and LA-MRSA ST398 has been found to be associated with human infections, including serious forms such as deep abscesses, cellulitis, necrotizing fasciitis and bacteremia (Pantosti, 2014).

Bovine Mastitis

Bovine mastitis mostly occur due to Staphylococci. Infections caused by methicillin-resistant staphylococci (MRS) are very difficult to treat posing public health risk. Devriese and co-workers (1972) first reported Methicillin-resistant *Staphylococcus aureus* (MRSA) in 5.2% of Belgian dairy farms MRSA became a sporadically reported finding from bovine mastitis there after spreading clonally. Some MRSA isolates from bovine mastitis are thought to be bovine and some of human origin. Clonal transmission between farmers and dairy cows has been shown to occur. More recently, livestock-associated MRSA (LA-MRSA) strains of MRSA ST398 and strains carrying MRSA mecC isolated from bovine mastitis cases also seen in humans.

In India Kumar *et al*. (2010) reported 10% prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) from 280 animals of Karan Fries (Taurus × Zebu) with mastitis.The same research team (Kumar *et al*., 2011) also reported Methicillin Resistant *Staphylococcus aureus* (MRSA) prevalence of 13% among 107 *Staphylococcus aureus* strains from cows with mastitis in a herd located in northwest India. Prevalence of 29% MRSA in the isolates of *Staphylococcus aureus* of bovine origin was observed in the state of Andhra Pradesh (Prashanth *et al.*, 2011). Genetic and evolutionary behaviour of *Staphylococcus aureus* associated

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with bovine subclinical mastitis using PFGE and spa typing in India revealed predominantly presence of spa types t267, t359 and t6877 (Mitra *et al.*, 2013).

Implications of LA-MRSA to Farmers and Veterinarians

1. Decreased treatment options – Penicillins, Cephems, Penems and Monobactams antimicrobial agents used in the treatment of Staphylococcal infections including mastitis shows resistance against MRSA making it useless. It may further lead to multi-drug resistance. Only enrofloxacin vancomycin, sulfa drugs, linezolid and cephalosporins with anti-MRSA activity like Ceftaroline and Ceftobiprole can be used but most of these antibiotics are not licensed for veterinary use.
2. Increase cost of treatment –Costly antibiotics further increases the cost of treatment.
3. Increased therapeutic failures – MRSA is resistant to antibiotics which are used as a first line of treatment. As infection progresses and fibrosis of the udder sets in, it leads to therapeutic failures.
4. Poor prognosis –MRSA often form biofilm and can adapt easily the udder leading to poor prognosis.
5. Occupational hazard – Some of the MRSA clones responsible for the mastitis in dairy cattle like CC398, CC97, CC 130 and CC151 are also transmissible to humans and are known to cause infections from abscess to infective endocarditis and bacteremia (Bardiau *et al*., 2013).

Control of Bovine Mastitis

Five Point Plan as recommended by National Mastitis Council -

1. Treat and record clinical cases
2. Post milking teat disinfection
3. Dry cow therapy
4. Cull chronic cases
5. Milk machine maintenance / Milkers' hygiene

Conclusion

Food producing animals are a primary source of newly emerging MRSA strains (livestock-associated MRSA [LA-MRSA]). In addition, humans (in particular farmers, farm co-workers, veterinarians etc) may represent an important source of new pathogenic strains affecting livestock. Once they have entered the food chain livestock may serve as a convenient vehicle for MRSA transmission possibly infecting food handlers and consumers. Due to their characteristic multi-host specificity, ST398 strains can represent an efficient vehicle of the multidrug-resistant determinants that are plasmid-encoded, favoring their transmission and spread. Regular surveillance of the microbiota in livestock and humans may facilitate the early identification of emergent MRSA clones with the capacity to transmit and cause disease among human populations.

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