STAReus MASTITIS:
STAPHYLOCOCCUS AUREUS MASTITIS:
WHAT WE NEED TO KNOW TO CONTROL THEM

Zecconi, A.

Department of Animal Pathology, Hygiene and Health. Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

INTRODUCTION

Among bacteria causing mastitis, only *Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma* species, and *Corynebacterium bovis* are considered as fully contagious. Among these, *S.aureus*, is currently the most frequently isolated contagious pathogen in subclinical and chronic bovine mastitis worldwide (1).

The reasons for the large spread of *S.aureus* intramammary infections (IMI) worldwide are obviously related to bacteria characteristics, but also to a general misunderstanding of the epidemiology of *S.aureus* IMI, leading to inefficient control measures.

The description of all the factors involved in *S.aureus* IMI should include many different aspects, and it is not possible to cover all of them in a single review. Therefore, in this paper we reviewed the main information useful to develop control programs for *S.aureus* IMI under field conditions and it is focused on the following topics: diagnosis, major risk factors, therapy, vaccination and control methods.

Bacteria characteristics

S.aureus are described " coagulase positive, β-haemolytic, maltose and mannitol fermenting organisms, forming pigmented colonies" (2). Not all strains of *S.aureus* have characteristics that are consistent with the previous description. Indeed, some *S.aureus* are α-haemolytic, β-haemolytic, α+β-haemolytic, δ-haemolytic, non-haemolytic and even coagulase negative (3).

To cause mastitis *S.aureus* initially must gain access to the mammary gland through the teat canal and then has to avoid removal by the flushing of the fluids during the milking process. Therefore, the ability to adhere to the epithelial cells and extracellular matrix (ECM) proteins is instrumental to colonize the gland and develop the pathologic process. The adhesion mechanism of *S.aureus* is complex and includes multiple proteins able to specifically recognize components of the microbial surface that recognize adhesive matrix molecules (MSCRAMM) (4), allowing bacterial anchorage in normal and inflamed tissues (5). Adhesive molecules are pivotal in the diffusion of *S.aureus* within and among herds, but they are only one of the several virulence factors involved in the pathogenesis of *S.aureus* infections. It is out of the scope of this review to describe all these virulence factors. However, assessing *S.aureus* genetic patterns is useful to understand its epidemiological features.

Several studies (6-8) indicate that virulence of *S.aureus* strains differ, an indication that strain typing is important. Similarly, transmissibility within a herd differs by strain type (9). Thus, the importance of evaluating the combination of *S.aureus* virulence factors has been recently emphasized both in human and veterinary medicine (8, 10, 11).

DIAGNOSIS

Several factors have been shown to influence accuracy for detecting *S.aureus* IMI using conventional bacteriological culture: i.e. sample type (quarter or composite), inoculum volume, sampling time and frequency. Hence, knowledge of the limits will guide the final decision on the method to be employed to detect *S.aureus* IMI at a cow or mammary quarter level.

It is generally accepted that there is relationship between bulk tank SCC (somatic cell count) and the proportion of cows with *S.aureus* in the herd (12). However, there is increasing evidence that a large proportion of cows in well-managed herds could be *S.aureus* positive, but with low SCC both at cow and bulk tank level (13, 14). This could be the result of recently acquired infections, reduced pro-inflammatory activity of strains or efficient host immune response (8, 15, 16).

Under field conditions, composite or quarter milk sampling can be applied. However, reliability of culturing composite milk samples compared with quarter samples for the diagnosis of *S.aureus* IMI 0.01 ml as inoculum volume showed a sensitivity and specificity respectively of 57.9 and 98% (17, 18). While composite milk sampling could be used for routine testing when the main interest is determining prevalence of infection at cow level, the use of this test in control programs, where the proper identification of infected cows is crucial, should be carefully evaluated.

Methods to increase diagnostic accuracy

One potential source of false positive results is the presence of irregular shedding of bacteria (19). However, recent studies suggested that increasing the inoculum volume to 0.1 ml will significantly reduce the risk of false negative results (20, 21).

These latter observations are supported by the studies on the accuracy of diagnosis based on different inoculum volumes. Direct plating of 0.01 ml of milk from established *S.aureus* infections failed to detect these organisms in about 10% of the samples, while plating 0.05 resulted in 6% of false negative samples (22). Further studies, using 0.01 ml as inoculum volume (23), found a 94.2% agreement between duplicate quarter milk samples positive for *S.aureus*. These results confirmed earlier reports and suggested that the single quarter sampling method was capable of identifying 82-94% *S.aureus* quarter infections (23).

To overcome some of the diagnostic problems, "augmented methods" have been also proposed. Among them, freezing-thawing and centrifugation are considered the most interesting. Freezing may affect viability of organisms contained in milk samples taken for bacteriologic culture but it could increase the sensitivity of the test breaking the clusters. However, (24) found that the presence of *S.aureus* in subclinical and clinical milk samples kept frozen at about -20ºC for 4, 6 and 16 weeks did not differ significantly. The centrifugation of milk samples has been proposed to overcome the low-shedding cases (25). The results showed that cultures of the sediment significantly increased the number of positive outcomes, in comparison with conventional methods.

ANALYTICAL METHODS

Milk culture could be performed either on blood-agar plate or using selective media such as Baird-Parker or a modification of the latter technique. However, the different phenotypes could cause several problems in diagnostic laboratories when selective media are applied. In our laboratory for an internal comparison, 50 confirmed *S.aureus* strains were tested on 3 different media: (blood-agar, Baird-Parker -BP- and Baird Parker supplemented with rabbit plasma and fibrinogen-BPR-PF). The results showed that 31/50 hadn't any surrounding clear zone on BP, 17/50 were coagulase negative on BPR-PF and 10/50 were only weakly positive in this latter medium. Therefore, milk culture on 5% blood agar plate still is the recommended method for *S.aureus* IMI diagnosis, followed by confirmation of the suspected colonies by other methods such as coagulase-test and biochemical tests. When selective media are applied, it is also recommended to confirm all the suspected colonies by other methods.

Independently from the method applied, identifying a single colony of *S.aureus* is enough to define the quarter (cow) as infected.

Very recently, methods based on Real Time PCR have been proposed to improve mastitis diagnosis, including for *S.aureus* (26). However, data available until now are not sufficient to evaluate if the molecular approach will be useful to improve the accuracy of current diagnostic methods for *S.aureus* IMI.

RISK FACTORS

Traditionally the prevalence of mastitis in heifers prepartum has been overlooked given the concept that heifers without a developed gland are not susceptible to IMI. Fox *et al.* (27) reviewed heifer mastitis, estimating prevalence between 1% and 10% of all heifers with *S.aureus* IMI at parturition. Therefore, heifers could be important risk factor acting both as a reservoir and as a vehicle for *S.aureus*.

Waage et al.(28) found that *S.aureus* was the major cause of clinical mastitis in heifers, with clinical mastitis noted during the first 2 weeks of lactation. Whether the heifer was raised on the farm or was imported into the herd did not influence the odds of having clinical mastitis in early lactation. In an extended study by (29), there was a significantly greater percentage of *S.aureus* isolated from epidermal and mucosal sites of heifers in high prevalence herds. Overall, heifers colonized with *S.aureus* in the mammary gland area were 3.4 times as likely to calve with *S.aureus* IMI, and this was the largest risk factor of disease.

Once *S.aureus* enters into the herd, the major vehicle of diffusion among cows is milking machines and improper milking procedures. Indeed, several studies found that *S.aureus* on milking unit liners could have the same fingerprint as the isolates causing IMI (30-32). Additionally, *S.aureus* has been isolated from udder cloths (33), thus implicating their role as a fomite in the spread of *S.aureus* mastitis.

Davidson (34) in large study on sites of colonization and IMI found the udder and teat skin to be an important reservoir associated with *S.aureus* mastitis. Zadoks et al. (35) would suggest that the teat skin is not a likely reservoir for *S.aureus* IMI. Such an argument was partially supported by (36), but it is opposite to that reported by (29, 31, 37).

Matos et al. (38) suggested that there are several other potential sources of *S.aureus* in a dairy, as this pathogen could be isolated from bedding and air of the parlour. Flies were not a source of *S.aureus* in this study, which is in contrast to that reported by (39). Roberson et al. (29) showed that in low-prevalence herds (<3%), bedding, insects, and water did not yield *S.aureus*, but high-prevalence herds did have some of these samples that were positive.

There is not a broad consensus on the role played by external sources in *S.aureus* IMI, out of milking machine and, potentially, by heifers. Therefore, these two factors are the most important ones we should be considered when we decide to apply a control program.

THERAPY

In practice, the common opinion that *S.aureus* has a relatively poor cure rate is based more on clinical impressions than on scientific evidences. Indeed, the cure rate for lactation therapy reported in literature has a very broad range (4-92%), a narrower, but still broad range has been reported also for dry-cow therapy (40). The recent debate on the increase in microbial resistance observed in human medicine, and particularly of methicillin-resistant *S.aureus* strains, increased the concerns on the use of antimicrobials for dairy cow therapy, particularly at drying-off. The discussion is still open, even if there are increasing evidences that the antibiotic therapy for mastitis and dry-cow treatment is not associated either with the development of methicillin-resistant strains or an increase of resistance (41). Despite this evidence, the application of antibiotic therapy following the principles of a prudent use of antibiotics, as proposed by various scientific organizations, should be recommended. To apply a prudent and efficacious protocol for mastitis treatment, different factors should be considered (42): type of pathogen involved, antibiotic

susceptibility patterns, severity of inflammatory response, duration of infection, stage of lactation and age of the cow.

Probably the most comprehensive analysis of the different factors affecting cure rate for clinical, subclinical mastitis and dry-cow therapy for *S.aureus* has been proposed by Sol and co-workers (43-45). Among the different factors, SCC showed to influence *S.aureus* cure rate after treatment of clinical and subclinical mastitis and at drying-off with lower cure rate when SCC were > 10^6/ml. Hind quarters showed a lower cure rate (subclinical and drying-off therapy), age and number of infected quarters showed to be significant only for drying-off therapy, with a decrease of the cure rate as age and number of infected quarters increased.

When the factors affecting cure rate are considered (i.e. age, SCC), the therapy is applied selecting molecules after susceptibility test and with a rational and efficient protocol, the cure rate could be higher than 75 % both for dry cow-therapy and for treatment of cows after calving (43-45).

VACCINATION

Much interest has been devoted to the development of a vaccine against bovine mastitis caused by *S.aureus,* but to date the results have not been fully convincing. Indeed, vaccination against *S.aureus* mastitis must take into account a large number of possible antigens and related interactions with the immune system. These different potential targets for an immune response are reviewed by (46) and these targets include capsules, adhesions, surface proteins and toxins.

Early vaccine formulations were composed of microorganisms that were cultured *in vitro*, killed, and injected systemically with or without toxoids and immunologic adjuvants as reviewed by (47, 48). Several such formulations were shown to increase the spontaneous cure rates of *S.aureus* IMI as well as to lessen the severity of infection but did not prevent new cases of mastitis, but none of them was broadly and consistently applied in dairy herds.

Heifer Vaccination

Nickerson et.al. (49) evaluated a polyvalent *S.aureus* vaccine in heifers beginning at 6 months of age (with periodic 6-month boosters) to determine if vaccination reduced prevalence of *S.aureus* mastitis during pregnancy and at calving. Results demonstrated that the percentage of new *S.aureus* infections during pregnancy was lower in vaccinates than controls (14.3 vs. 25.9%), the percentage of quarters showing chronic *S.aureus* infection was lower in vaccinates than controls (10.7 vs. 18.8%), and at freshening, the percentage of quarters infected, with *S.aureus* was lower in vaccinates than controls (8.9 vs. 16.1%). The data demonstrated a positive effect of vaccination in increasing antistaphylococcal antibody titers and in preventing new *S.aureus* infections when the program was initiated at an early age in heifers raised in a herd with high exposure to this mastitis pathogen.

Edinger et al. (50) developed a herd-specific vaccine based on two strains of *S.aureus* previously isolated from cases of clinical mastitis in the herd. Results showed that prevalence of *S.aureus* in quarter milk samples taken at calving and three to four weeks post partum did not differ significantly between the vaccine and control group. Regarding the development of clinical mastitis during the first three months after calving and the prevalence of *S.aureus* in quarter milk samples taken before the onset of treatment, there were no significant differences between the groups. The SCC was lower in vaccinated than in control heifers. However, the difference was only significant on the third milk test day.

Cow Vaccination

In dairy cows, one of the first vaccines developed was a heat-killed capsular type A and B *S. aureus* strains and capsular polysaccharide, and it was used in two herds. The results showed that the vaccinated animals had a decrease in mastitis incidence and higher milk yield in comparison with unvaccinated herd-mates (51). A vaccine containing whole inactivated bacteria with pseudocapsule and α-β toxoid was used in a field trial in Norway (52). The study showed that a significant increase in antibody was observed only for pseudocapsule and α toxoid. Moreover, vaccinated heifers showed a prevalence of *S.aureus* mastitis of 8.6%, while in control heifers it was of 16%.

One of the largest field trials on *S.aureus* vaccine efficacy was reported by (53). Results showed no overall reduction in the incidence of clinical mastitis, even though differences were observed within individual herds. Overall, the known commercially available *S.aureus* vaccines have shown limited efficacy under field conditions. A vaccine which would prevent the occurrence of bovine mastitis caused by *S.aureus* or a vaccine that would augment *S.aureus* control would be of benefit. More recently, two vaccines were commercially available in some countries, but only for one of these two data are available (54-56). This patented vaccine is derived from 3 field strains of *S.aureus* which contain a broad spectrum of antigenic and immunogenic properties. The composition enables the vaccine to induce a strong homologous as well as heterologous immune response. The results of the field trials showed a 70% specific protection from infection and almost complete protection from udder inflammation expressed by the low SCC (100×10^3 cell/ml) of the vaccinated cows. The effect of vaccination on subclinical udder infection revealed a significant clearance of *S.aureus* udder infection in comparison to the control, which was expressed also by a reduction in SCC to normal range. Because of low rate of spontaneous infection among the heifers in the field trial, no specific protection could be evaluated. However, a significant difference in SCC between the vaccinated and nonvaccinated heifers was found (108×10^3 and 178×10^3 cell/ml respectively). A significant difference in milk production (0.5 Kg per cow per day) was found as well.

Innovative vaccines developed by the means of molecular methods have been investigated by several research groups (57-60). Results are very encouraging, but none of these vaccines is available in dairy field.

Thus, even if *S.aureus* vaccine was the object of a large

number of studies, still a vaccine with proven efficacy in commercial dairy herds and in different areas is not available.

CONTROL PROGRAM

Environmental sources and non-dairy animals do not appear to be significant reservoirs and vectors for the disease. Therefore, a control/eradication program for this pathogen could be hypothesized. The frequency of infected herds and cows, and the high cost of the disease represent the reason to develop control programs. Moreover, the availability of accurate diagnostic procedures and efficacious therapeutical protocols allow for their implementation.

To develop a strategy to control the infection, the approaches currently applied are the test-and-cull strategy and control programs based on segregation. Culling is often suggested as the only way to control *S.aureus* (61, 62). However, there is poor scientific evidence either on the efficacy or on the economic return of this approach. Indeed, (63) showed that at the end of a program based on test-and-cull strategies applied in three herds, all the three herds had a very similar culling rate, but only the third one, applying a well-managed program in addition, achieved the control of *S.aureus* IMI. Therefore, culling could be a component of a control program, mainly as the most efficient way to remove the chronic *S.aureus* cows. However, as a main method of control it showed to be poorly efficient and with a negative economic impact.

Control programs based on segregation followed the general principles of contagious mastitis control (64) and was based on precise diagnostic procedures and strict control of segregation of infected cows.

We reported the epidemiologic pattern IMI in 9 commercial dairy herds after establishment of a standardized and detailed mastitis control program (65).

The main steps in the control program are (Figure 1):

1. Application of a precise and consistent milking procedure that included use of a single-service towel to clean the teat, forestripping, and use of postmilking teat disinfectants of known efficacy.

2. Establishment of a milking sequence to reduce infection risk, by milking healthy cows first, then cows and heifers that had recently entered the herd either through purchase or freshening, and then milking cows with *S.aureus* IMI last.

3. After the first sampling of all lactating cows at the time of enrolment to segregate infected cows, a precise sampling schedule is used. Purchased cows are sampled 5-7 and 10-14 days after entry into the herd, and cows that had recently calved are sampled 5-7 and 10-14 days after calving. Cows with *S.aureus* IMI are segregated and are not sampled again until they have calved. Non-infected cows are sampled again 2, 4, 7, 10, 14 and 18 months after the first sampling.

4. Diagnosis of *S.aureus* IMI is performed with mammary quarter milk samples by bacteriologic culture on 5% blood-agar media. Quarter samples are collected to increase the sensitivity of detection. Recovery of a single colony of *S.aureus* is considered a positive result indicating an IMI. All

mammary quarters of all cows are treated at drying-off with a commercial antimicrobial treatment. Choice of the product is based on the susceptibility of the *S.aureus* strains isolated in the herd as determined by use of the disc diffusion method.

5. Treatment of infected lactating cows without clinical signs is restricted to those with ≤ 3 lactations and in their first 30 days of lactation, to avoid treatments with a poor cure rate. These cows are moved to a hospital pen to be sampled 5-7 and 10-14 days after the end of the antimicrobial withdrawal period. Only cows that are judged cured because of negative results of 2 consecutive bacteriologic cultures of milk samples are allowed to leave the hospital pen.

6. Antimicrobial and anti-inflammatory treatments are administered to cows that developed clinical mastitis, independently of their *S.aureus* infection status, and these cows are moved to the hospital pen to be sampled 7 and 14 days after the end of the withdrawal period. Only cows that were judged cured are allowed to leave the hospital pen to come back to the group of origin.

7. At the herd level, farm managers are advised to improve and keep proper bedding hygiene.

8. The control program is monitored directly by means of monthly visits by a trained practitioner and discussion of analysis results, and indirectly by a weekly check of samples sent to the laboratory, to ensure compliance.

Results of this field study suggested that a very low incidence rate can be achieved after about 10 months of the control program. Young cows and freshening cows are the most likely to develop new IMI among uninfected cows when segregation of infected cattle is used; uninfected cattle should be carefully checked and specific procedures should be applied to reduced the risk of infection. It could be suggested that heifers should be housed separately from older cows for at least 2 weeks before calving, to reduce the risk of becoming infected from older cows during periparturient period.

The proposed control/eradication program based on segregation showed, on average, to reduce the incidence rate of *S.aureus* IMI below 2% in 10 months and <1% in 18 months. Cost/benefit analysis showed that a positive economic return could be achieved even when starting prevalence of *S.aureus* IMI are in the range 10-20% (66).

CONCLUSIONS

Programs based on segregation has been applied, but on a smaller scale when compared to *Str.agalactiae*. Diagnostic problems, poor cure rate, and unknown sources of infection are often the arguments advocated to refute the application of a control program. Moreover, the presence of intramammary infections, without large increase of SCC induces many practitioner and farmers to underestimate the impact on milk quality and yield.

However, based on the available scientific and practical evidences we can reasonably affirm that:

- *S.aureus* has a variable, but large economic impact on

dairy herd;

- Diagnosis of *S.aureus* IMI is feasible with conventional methods by experienced laboratories;
- Well-designed *S.aureus* therapy protocols have a cure-rate not inferior to the one observed against other intramammary pathogens;
- *S.aureus* is not an obligate parasite of the mammary gland, but the importance of potential reservoirs on other body sites or in the environment significantly decreases as the prevalence of IMI decreases in the herd. Therefore, they will not affect the control programs.

Therefore, when control programs are based on few important points such as the isolation or removal of reservoirs, the avoidance of *S.aureus* transmission during milking, a careful monitoring by a trained practitioner, they will be successful with positive economic returns for both the farmer and the practitioner.

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FIGURE

Figure 1 - Control program scheme