Surveillance of antimicrobial resistance of maltose negative *Staphylococcus aureus* in South African dairy herds

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Abstract

Background

The discovery of antimicrobials in the 1930s was one of the greatest achievements in medicine. However, bacterial resistance to antimicrobials was already observed in the 1940s and has been reported since then in both human and veterinary medicine, including in dairy cows. Many years of monitoring milk samples in South Africa, has led to the identification of a new strain of *Staphylococcus aureus* (*S. aureus)*, which is maltose negative and appears to be an emerging pathogen. In this study the differences in susceptibility to antimicrobials of this strain were evaluated over time, over different seasons, in different provinces, and according to somatic cell count (SCC) categories.

Results

A data set of 271 maltose negative *S. aureus* isolates, cultured from milk samples from 117 herds out of the estimated 2000 commercial dairy herds in South Africa between 2010 and 2017, was studied using the disc diffusion method. This analysis was done using the Clinical Laboratory Standards Institute (CLSI) breakpoints in order to compare using both the previously used system (intermediate category grouped with resistant) and more recent system, (intermediate category grouped with susceptible). The results between the previously used system and the more recent system analysis differed for tylosin, cefalonium, oxy-tetracycline and cloxacillin. Neither the analysis using the previous system nor the more recent system showed an effect of province for the maltose negative *S. aureus*. This was in contrast to the results for maltose positive *S. aureus* where differences between provinces were shown in a previous study. For the susceptibility testing of 57 maltose negative *S. aureus* and 57 maltose positive *S. aureus* the minimum inhibitory concentration (MIC) results for the maltose negative *S. aureus* confirmed the results of the disc diffusion method.

Conclusions

The maltose negative strains of *S. aureus* differed in general, in their antimicrobial resistance patterns over time, in comparison to maltose-positive *S. aureus* strains. MIC testing also indicated that more multi-resistant isolates were seen with the maltose negative *S. aureus* than in the maltose positive strains.

Background

The genus *Staphylococcus* consists of a variety of opportunistic pathogens of variable relevance in veterinary medicine. The most clinically relevant staphylococci in veterinary medicine are the coagulase positive *Staphylococcus aureus* and members of the *S. intermedius* group (SIG) [29], particularly *Staphylococcus pseudintermedius (S. pseudintermedius)*. A noted property of staphylococci is their ability to become resistant to antimicrobials. Methicillin resistance is of particular relevance, because it is conferred by a presence of the mecA gene, which encodes for the production of an altered penicillin binding protein (PBP) (PBP2a or PBP2') that has a low affinity for all beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems) [17]. Methicillin-resistant *S. aureus* (MRSA) is recognised as a significant problem in human medicine and it is among the most important infections in hospitalized individuals and in people in general [16].

In veterinary medicine *S. aureus* (maltose positive) is the biggest problem in the dairy industry in South Africa and globally [24]. The infected udder is considered the primary reservoir of *S. aureus* and the organism is believed to be transmitted during milking. Despite this, a proportion of heifers which are already infected with *S. aureus,* enter the milking herd [23]. This suggests routes of transmission in addition to the milking equipment and the milking parlour. A good understanding of *S. aureus* reservoirs and transmission is essential for the effective control of the organism in a herd. The probability of treatment resulting in cure of *S. aureus* (maltose positive) infection is calculated by taking the following factors into account: parity; the stage of lactation; the SCC level; the specific teat position on the udder; the number of quarters infected; and the duration of treatment required [32].

The Milk Laboratory at the Faculty of Veterinary Science at the University of Pretoria has provided an extensive dairy cow udder monitoring programme in South Africa. Since 2005, an increasing number of coagulase positive, maltose negative staphylococci have been isolated, that were confirmed as maltose negative *S. aureus* by molecular methods. These organisms were first identified from a dairy cow in a single South African dairy herd with a somatic cell count of less than 100 000 cell/ml of milk. As early as three years later these organisms were isolated from numerous other dairy herds in South Africa, albeit with effective susceptibility to antimicrobials that were tested routinely and with a low SCC. However in more recent years 2016/2017/2018, coagulase positive and maltose negative staphylococci were isolated showing resistance (MRSA, cefoxitin disc) where some of the milk samples started to show higher SCC (> 400 000cells/ml milk) than initially shown (personal experience). In addition to the previous study which attempted to characterise this emerging pathogen, a further evaluation was carried out of the resistance trends evident in historic disc diffusion susceptibility data and more recent MIC data.

The main objective of this study was to investigate the retrospective (disc diffusion) antimicrobial surveillance data of maltose negative *S. aureus* in different provinces, seasons and SCC categories, and to determine any differences between the use of the previous versus the most recent CLSI classification systems. An additional objective was to compare the MIC results of maltose positive and maltose negative *S. aureus* (emerging pathogen) and to discuss the practical implications thereof.

Results

The first part of this study was the retrospective data analysis (disc diffusion) which was done only on maltose negative *S. aureus*, as a previous study had been done with a similar analysis of maltose positive *S. aureus* that combined the resistant and intermediate results and reported susceptible results separately [14]. The eight antimicrobials that were used in this retrospective study were the commonly used antimicrobials that are available as intramammary remedies in South Africa. The original classification [6; 7] and the more recent classification (intermediate grouped with susceptible) [8; 9] of antimicrobial resistance showed similar trends. These trends of resistance to ampicillin, cephalexin, cefalonium, cloxacillin, oxy-tetracycline and penicillin

peaked (at highest) in 2011, and for tylosin in 2013, and then decreased over time. However, in 2014 there was a slight increase in antimicrobial resistance seen for cloxacillin and in 2016 for ampicillin, cephalexin, cefalonium, penicillin and tylosin (Figures 1, 2, 3, 4, 5 & 6). The analysis of antimicrobial resistance for cloxacillin showed significant differences between SCC categories only according to the GLMM analysis ($p<0.05$). Cefoxitin, cloxacillin and cefuroxime showed no significant differences for years and provinces (Table 1). The analysis of antimicrobial resistance of maltose negative *S. aureus* for oxy-tetracycline, cephalexin, ampicillin, tylosin, cefalonium and penicillin G showed significant differences according to the GLMM ($p<0.05$) over time (years). Similar apparent trends (but not significant) were shown for clindamycin which was used from 2009 to 2012 and an apparent increase in percentage of antimicrobial resistance and (also not significant) for cefoxitin which was used from 2014 to 2017 and showed a decreased antimicrobial resistance over time.

These data were re-evaluated according to the more recent classification system [8; 9] that grouped results from susceptible and intermediate data together, so that the resistant data were reported separately. Some differences from the previous analysis were apparent as a detailed examination of Tables 1 and 2 will show. Tylosin, cloxacillin, oxy-tetracycline and cefalonium showed differences in the effects of year, season, province and SCC categories on antimicrobial resistance as seen between the original system of classification (Table 1) compared to the more recent system of classification (Table 2) using the CLSI guidelines. According to the new guidelines, tylosin, oxy-tetracycline and cefalonium showed no significant effects at all on antimicrobial resistance from the factors measured (Table 2), whereas they had showed some effects of year, season and SCC categories when using the previous classification (Table 1). Cloxacillin showed an effect of SCC category only when using the previous classification system for analysis (Table 1), but there appeared to be possibly a slight effect of season as well as when using the more recent classification (Table 2). There was no effect of province on antimicrobial resistance when using both the previous system (Table 1) and the more recent system (Table 2) of the CLSI guidelines. Ampicillin, penicillin G, cefoxitin and cefuroxime showed similar effects of year, province, season and SCC categories for both the previous system (Table 1) and the more recent system (Table 2) CLSI guidelines. For ampicillin, comparing the results from the previous system of analysis, seasonal effects showed differences only for autumn compared to spring (Table 1). However, by using the more recent system, the results for ampicillin appeared to show an effect of both summer and autumn (Table 2) with high resistance at those times, and this could be compared to spring where there was low resistance.

This comparison between the analysis of the effects of year, season, province and SCC categories of antimicrobial resistance of maltose negative *S. aureus* shows that the new breakpoints (CLSI) as well as the grouping of intermediate readings with susceptible readings instead of with resistant readings, as was done in the past, does indeed make a difference to the results (Tables 1 & 2). The logic behind the change of this grouping, was that in the past, intermediate readings were grouped with resistant readings to create more strictly defined categories.

Antimicrobial products shown in Tables 3 and 4 are all the products from the PM 32 panel (Beckman Coulter) that showed resistance to any of the isolates tested. The 57 maltose positive and the 57 maltose negative *S. aureus* isolates were all resistant to ampicillin. Out of the total of 114 isolates overall, only 37 were resistant to more than one product, 30 maltose negative *S. aureus* and seven maltose positive *S. aureus* (Table 4). There were a total of 25 multidrug resistant (MDR) isolates (isolates resistant to an antimicrobial from three or more antimicrobial classes), 3 maltose positive and 22 maltose negative *S. aureus* (Table 4).

Discussion

This study on the maltose negative *S. aureus* showed no significant differences of antimicrobial resistance between the provinces, and only a limited significant difference related to seasons and SCC categories, whereas there were no significant interactions between any of the variables considered (Tables 1 & 2). The relationship of the occurrence of SCC category to antimicrobial resistance was in agreement with a study in Denmark [5], which also found high SCC to correspond with low antimicrobial resistance and low SCC to correspond with high antimicrobial resistance. This could be due to the SCC being more of an indicator of irritation and severity of the infection rather than an indicator of antimicrobial resistance of the organism.

In contrast, the study on maltose positive *S. aureus* showed that when considering the provinces, the lowest prevalence of antimicrobial resistance to the majority of the categories of antimicrobials that were tested was present in KwaZulu-Natal during spring, except for cephalosporins which had the lowest levels of prevalence of bacterial resistance in Gauteng during winter [15]. Although, there were great differences in the numbers of herds and samples between the provinces, these differences were taken into account in the model, during the analysis.

Resistance patterns of the maltose positive *S. aureus* to the eight antimicrobials varied in the different seasons and provinces, possibly because of the different weather conditions, as well as the action and spectrum of antimicrobials [15]. There was one specific strain of maltose negative *S. aureus* identified, originating in and found mostly in KwaZulu Natal, but also now present in all nine provinces of South Africa, albeit in small numbers.

The antimicrobial resistance trends (Figures 1 to 6) were in agreement with those shown for the same antimicrobials with coagulase negative staphylococci [27], but in contrast to the trends shown for the maltose positive *S. aureus* over time [14]. The study on the maltose positive *S. aureus* showed a general increase in resistance over time except for the 20 well managed herds (part of the pro-active udder health programme), which showed a decrease in resistance over time [14]. However, due to the general increase in antimicrobial resistance in recent years, there were some cases where there may not have been any effective products available and then an intermediate product would have been used at a higher concentration, which is why the intermediate responses should now be grouped with the susceptible responses. However, the trends of antimicrobial resistance over time of maltose negative *S. aureus* evaluated with both the previous (intermediate responses grouped with resistant responses) (Figures 1, 2 & 3) and the more recent system (intermediate responses grouped with susceptible responses) have been similar for these organisms tested (Figures 4, 5 & 6).

The antimicrobial resistance trends (Figures 1 to 6) and profiles (Tables 3 & 4), allow for informed treatment choices to be made without the need to wait for any specific antimicrobial sensitivity test results. However, the results of antimicrobial sensitivity tests (disc diffusion or MIC), are just an indication that the particular organism has the ability to be killed by a particular antimicrobial *(in-vitro)*. In the udder, the situation may be very different from tests *in vitro*. This is

because the site of the infection may be very difficult to reach via very small arteries and lactiferous ducts and also due to udder pharmacodynamics (very few products are successful in a water and fat environment). As a result of this, the treatment success for mastitis in is not very high (27%) as has been determined in a large study done in five European countries; France, Hungary, Italy, the Netherlands, and the United Kingdom [33] and can lead to the development of antimicrobial resistance by mastitis-causing organisms. Treatment success against *S. aureus* tends to be better in the dry period but still not ideal [3], therefore the pro-active udder health programme should be applied. This is why as far as mastitis is concerned, the focus should be on the prevention and monitoring through the pro-active udder health programme [25] rather than on the actual treatment.

The second part of this study concerned the MIC test on 57 maltose negative and 57 maltose positive *S. aureus* isolates. The MIC results of antimicrobial resistance (Table 3) confirmed the disc diffusion results (Tables 1 & 2) for maltose negative *S. aureus* for the products tested. Antimicrobials are grouped into three broad groups that are based on approved usage (US FDA): approved for human use only; approved for animal use only; or approved for the use in both humans and animals. The antimicrobials studied for the retrospective data analysis for the maltose negative *S. aureus* are from the category approved for the use in both humans and animals. However, from the antimicrobials that were studied for the MIC testing (PM 32 panel), daptomycin, ertapenem, fosfomycin, meropenem, moxifloxacin, rifampin, teicoplanin and tobramycin are approved for human use only and the remainder of antimicrobials in the panel (PM 32) are approved for both use in humans and animals (Tables 3 & 4). Antimicrobials that are approved for human use only create a reserve of unique antimicrobials for humans. Antimicrobials that are approved for animal use only, such as the ionophores, should not create a risk to human health.

Antimicrobials contribute to animal care in four ways: to treat animals diagnosed with an illness; to control spread of illness within a herd or flock; to prevent illness in healthy animals when exposure is likely; and to ensure healthy growth by maintaining the right balance of bacteria for improved nutrient utilization (antimicrobials approved for animal use only) [10]. Antimicrobials that are used in animals are important for global food security. Estimates have been made that nearly one billion people in the world do not get enough to eat each day and nearly three billion are trying to diversify their diet to include more meat, milk and eggs [10]. Veterinary medicines including the antimicrobials provide a valuable tool to help veterinarians and producers to deliver healthy animals to meet the growing need for safe, nutritious, affordable food, while making the most of limited natural resources in a sustainable manner. As the world population is expected to grow to nine billion by 2050, tools such as antimicrobials which keep animals healthy will be essential to meet the increasing demand for food [10].

Antimicrobials that could be considered for routine testing by veterinary diagnostic laboratories, may be divided into four groups [11]:

Group A: Antimicrobials with specific interpretive criteria for veterinary medicine;

Group B: CLSI approved interpretive criteria for human medicine;

Group C: No veterinary species-specific or human-specific interpretive criteria;

Group D: Supplemental to be tested selectively.

Ampicillin, oxacillin, erythromycin, penicillin and tetracycline are the corresponding antimicrobials from the panel tested which are approved for the control of bovine mastitis specifically.

The distribution of the MIC test results of the antimicrobials tested are summarized in Tables 3 and 4 for maltose positive and maltose negative *S. aureus* isolates respectively. One maltose positive and 12 maltose negative *S. aureus* isolates were resistant to oxacillin (Table 4). For erythromycin one maltose positive and nine maltose negative *S. aureus* isolates proved to be resistant, respectively. The MICs of clindamycin were two dilution steps lower (Table 4) than those of azithromycin, cefotaxime, erythromycin, gentamycin, linezolid, teicoplanin, tetracycline, tobramycin, trimethoprim / sulphamethoxazole and vancomycin for maltose negative *S. aureus* isolates (Table 3). However these patterns differed for the maltose positive *S. aureus* isolates (Table 3). In general, the resistance rates of the maltose positive *S. aureus* obtained in this study, corresponded well to those reported in other studies [28; 35].

The *MIC* 50 represents the *MIC* value at which ≥*50% of* the isolates *in a* test population *are* inhibited, and it *is* equivalent to the median *MIC* value. The MIC 90 represents the MIC value at which ≥90% of the isolates in the test population are inhibited [30]. The MIC breakpoints (chosen concentration [mg/L] of an antimicrobial which defines whether a species of bacteria is susceptible or resistant to the antimicrobial) of certain antimicrobials were susceptible for MIC 50 and MIC 90 for both the maltose negative and the maltose positive *S. aureus*, except for MIC 90 of maltose negative *S. aureus* (Table 3). The maltose negative *S. aureus* was more resistant to the amoxicillin clavulanic acid combination (used in human medicine), ampicillin and cefuroxime at MIC 90, and for clindamycin at MIC 50 (Table 3). However, oxacillin was more resistant for maltose positive *S. aureus* for MIC 90 and MIC 50 and clindamycin for MIC 90 (Table 3). Infrequently found resistance patterns were found in 17 of the 57 maltose negative *S. aureus* isolates which were resistant to vancomycin and one maltose positive and eight maltose negative *S. aureus* isolates which were resistant to oxacillin [11].

Overall in this study there were more multi-drug resistant maltose negative *S. aureus* than maltose positive *S. aureus* isolates and the same general interpretation applied for isolates resistant to two or more antimicrobials of varying combinations in general (Table 4).

There have been many studies in both animal and human medicine that have identified multidrug resistant and pan-drug resistant *S. aureus* isolates [12]. However, most of these studies were done on traditionally identified coagulase positive, maltose positive *S. aureus*, since the maltose negative coagulase positive isolates were thought at that time to have been part of the *S. intermedius* group of isolates. Multi-drug resistant *S. intermedius* and *S. pseudintermedius* isolates have been described in dogs, cats and horses, mainly from skin infections [20; 36]. Although a coagulase positive, maltose negative *S. aureus* strain was subsequently isolated from bovine mastitis [13], there appear to be no antimicrobial susceptibility profiles of this organism yet. The maltose negative *S. aureus* in this study was originally phenotypically identified as *S. pseudintermedius* [11; 29], but further MALDI-TOF MS and 16S r RNA sequence analysis on these isolates from dairy cattle, showed that these were in fact a strain of *S. aureus* (Karzis et al. submitted JDS 2019). Therefore it

would make sense that these maltose negative *S. aureus* isolates in this study, have reacted in a way more like the classical *S. pseudintermedius* isolates in other studies, identified from other species [20; 36]. One of the limitations of this study is the limited sample numbers of maltose negative *S. aureus* available to be used for this trial.

Human nasal *S. aureus* colonization has previously been reported as being a risk factor for pig farming [4] and *S. aureus* strains from pig farmers were found to be those present in pigs but had not been found in non-farmers [2]. However, little attention was paid to MRSA in pigs until 'unexpected' MRSA infection and colonization were identified in people that had been in contact with pigs in the Netherlands [34]. In the light of this research in pigs, it is possible that in a similar way of transmission of some strains of these resistant *S. aureus* (predominantly maltose negative strains) isolated from dairy cattle in South Africa could be from people. Previous studies done in KwaZulu Natal [31], have indicated zooanthroponosis ("reverse zoonosis") of *S. aureus* in South Africa, with one of the strains identified as the same maltose negative *S. aureus* strain. These maltose negative strains of *S. aureus* seem to have completely different antimicrobial resistance trends and severity of antimicrobial resistance, when compared to the traditionally identified maltose positive *S. aureus* and thus need to be treated differently in practice.

Resistance to antimicrobial agents, such as doxycycline and trimethoprim-sulfamethoxazole is very uncommon in *S. aureus.* In this study the MIC 90 of trimethoprim-sulfamethoxazole was the same for both the maltose negative and the maltose positive *S. aureus*. The reason for these uncommon resistance profiles of maltose negative *S. aureus* isolates, which have also shown to be resistant to antimicrobials that are only used in human medicine, (e.g. carbapenems like imipenem and ertapenem) which had 10 resistant isolates each (Table 4), remains questionable. These are antimicrobials which are not used at all in animal medicine. Zooanthroponosis seems to be a strong possibility, because these isolates have been shown to be present on the skin of humans that come into close contact with dairy cattle. This would be similar to the findings of the studies with the dogs in Brazil [19] and pigs in Germany [2] and the Netherlands [35] respectively. Further studies need to be done to explore the origin of such resistant isolates of maltose negative *S. aureus*. Future work is also necessary to determine the resistance genes present in resistant maltose negative *S. aureus* strains.

Similarly to this study, a study done in dairy cattle in Tennessee also found that there was a variation of prevalence of antimicrobial resistance of *S. aureus* within and among farms over time, with an increasing trend in tetracycline resistance [1]. This was also in accordance with the report which showed that the predominant antimicrobial groups used in animal health management in South Africa from 2014 to 2015, were the growth promoters (animal-use-only antimicrobials) (62%) followed by tetracyclines (17%) and macrolides (11%) [21]. In this study five isolates were resistant to tetracyclines and ten isolates were resistant to macrolides (represented by erythromycin) and only one of each of these isolates were maltose positive, whereas the rest were maltose negative *S. aureus* (Table 4).

The use of antimicrobials in South Africa in 2015, has been stated to be 21 149 standard units per 1000/population (IMS Health 2015) (Note: 1 standard unit is equivalent to 1 tablet, or injection), and this was significantly higher than most other countries in the world. Broad-spectrum penicillin usage in humans in South Africa was 1.3 to 3.3 times more than that used in other countries and 0.8 times that used in the United Kingdom or the USA [21] It is by no means clear if the high number of maltose negative *S. aureus* isolates resistant to penicillin and ampicillin (Table 4) might solely be attributed to the high antimicrobial usage.

Conclusion

The antimicrobial resistance trends found were similar for both the previous system (intermediate responses grouped with resistant responses) and the more recent system (intermediate responses grouped with susceptible responses) of analyses. These trends for ampicillin, cephalexin, cefalonium, cloxacillin, oxy-tetracycline and penicillin peaked (at highest) in 2011 and for tylosin in 2013 and then decreased again. These antimicrobial resistance trends over time, showed a closer comparison with analysis of similar data for coagulase negative staphylococci than for the maltose positive *S. aureus*. Antimicrobial resistance trends over time also differed between maltose negative and maltose positive *S. aureus*. Antimicrobial resistance of maltose negative *S. aureus* showed no significant differences between provinces (using both the previous and also the more recent interpretation) and there were only limited differences between seasons for ampicillin, penicillin, tylosin and cefalonium (using the more recent interpretation system). For cloxacillin and cefalonium specifically, high SCC corresponded with low antimicrobial resistance and were significantly different from low SCC which to corresponded with high antimicrobial resistance. The MIC results of antimicrobial resistance for maltose negative *S. aureus* confirmed the results of the disc diffusion method. The results of the MIC method also showed more resistance in general for maltose negative than for the maltose positive *S. aureus* isolates to most of the antimicrobials that were used. The MIC breakpoints were susceptible at MIC 50 and MIC 90 for both maltose negative and maltose positive *S. aureus*, with the exception of maltose negative *S. aureus* at MIC 90.

Methods

Data collection and bacterial identification

In an initial retrospective study 271 isolates from 117 dairy herds out of approximately 2000 commercial dairies in South Africa [18] were analysed from 2010 to 2017. These herds were located in all nine provinces of South Africa but with greatly varying numbers in the different provinces, namely: Gauteng (n= 8), KwaZulu Natal (n= 170), Free State (n= 4), Eastern Cape (n= 56), Western Cape (n= 27), Northern Cape (n= 1), North West (n= 1), Limpopo (North) (n= 1) and Mpumalanga (n= 3). All herds from which these isolates were collected participated in the pro-active udder health programme (routine testing of microbiology and cytology) at the milk laboratory [14]. The milk samples were collected in an aseptic manner according to a standard operating procedure [22]. These isolates were collected and cultured according to the method recommended by the National Mastitis Council [22] and somatic cell counts were performed using a Fossomatic 5000 and Fossomatic FC (Rhine Ruhr). Isolates to be tested for routine antimicrobial susceptibility were all selected from milk samples with a somatic cell count (SCC) (Fossomatic 5000 and Fossomatic FC, Rhine Ruhr) of more than 400 000 cells/ml [23], when applicable, in order to include

cases of subclinical mastitis. This was the general rule for routine antimicrobial susceptibility testing. However, when a maltose negative *S. aureus* was isolated from a herd, antimicrobial sensitivity testing was done on that organism regardless of SCC. Phenotypic differentiation of staphylococci was initially identified based on colony morphology, pigmentation, haemolysis, catalase, staphylase, maltose and potassium hydroxide tests that were used [26]. A positive maltose agar reaction confirmed *S. aureus* and a negative maltose reaction confirmed an organism potentially from the *S. intermedius* group [11; 29].

Antimicrobial susceptibility testing

Disc diffusion method

The disc diffusion method [4] was used to determine the antimicrobial susceptibility of the routine diagnostic samples that were used for the retrospective data analysis. The initial results were based on the diameter of the inhibition zones and were classified as sensitive, intermediate or resistant (intermediate grouped with resistant) in accordance with the clinical breakpoints that were established by the CLSI [6; 7] (previous system). These data were later reclassified according to the CLSI [8; 9] (intermediate grouped with susceptible), and the results were compared.

Nine antimicrobials used in intramammary treatment (dry and lactating remedies) that were available for use in South Africa were tested. These were the penicillins (ampicillin 10 µg, cloxacillin 5 µg, penicillin G 10 IU), cephalosporins (cephalexin 30 µg, cefuroxime 30 µg), lincosamides (clindamycin 10 µg), tetracyclines (oxy-tetracycline 30 µg) and macrolides (tylosin 30 µg) and (cefoxitin 30 µg). The lactating cow numbers of the herds in this study varied from approximately 30 (smallest herd) to 1 700 cows, (largest herd) [18].

MIC method

In addition, MIC tests were performed on 57 coagulase positive, maltose negative *S. aureus* isolates (2012-2013 n=15; 2018-2019 n=42) and 57 maltose positive *S. aureus* isolates (2012-2014 n=11; 2017-2018 n= 46), isolated from 38 dairy herds mainly from KwaZulu Natal and Eastern Cape provinces, but also with a few samples from Western Cape, Gauteng and Mpumalanga. The 57 maltose negative *S. aureus* isolates were the total number of these isolates collected during the time periods mentioned. For the maltose positive *S. aureus* isolates, the same number (n = 57) of isolates from the same farms, with similar corresponding SCC ranges and from similar time period to those of the maltose negative *S. aureus* samples were selected. Antimicrobial agents that were selected were based upon the availability of commercial intramammary infusion products or as representatives of their respective antimicrobial classes such as: ampicillin, oxacillin, erythromycin, penicillin and tetracycline.

The MIC were determined by using the automated broth microdilution method (PM 32 panels and Microscan 40 Walkaway system, Beckman Coulter, California, USA) and the results were evaluated according to CLSI [8; 9].*Staphylococcus aureus* ATCC 25923 and *S. pseudintermedius* ATCC 49444 served as the reference strains for quality control purposes. The MIC data analysis used the LabPro software of the Microscan 40 Walkaway system, (Beckman Coulter, California, USA) in order to determine the MIC 90 and the MIC 50 was calculated manually.

Statistical Analysis

The retrospective data were originally analysed according to the previous conventional system [6; 7], as described above. However, after the introduction of the new CLSI [8; 9] recommendations, this analysis was repeated using the recently introduced system [8; 9], and the two sets of results were compared.

The SCC categories that were used were, low (< 150 $x10^3$ cells per ml milk), medium (150 $x10^3$ to 400 $x10^3$ cells per ml milk) and high (> 400 $x10^3$ cells per ml milk).

The Chi-square test was used to check for the existence of any effect of year, province, season or SCC category on the response variable, the resistance to the different antimicrobials. This Chi-square test also allowed classification of the categories of each variable in order to introduce the one with the lowest level of resistance as the reference category in the following GLMM (general linear mixed model) analysis. Apparent relationships between season, province and SCC category on the prevalence of antimicrobial resistance of all *S. aureus* isolates were tested in a multivariate model, a ('glmer' function within 'lme4' package of the R software © version 3.3.3), with a 95% confidence interval using a logit link-function. This model allowed the analysis to take into account the random effect from the different herds. This type of general linear mixed model (GLMM) takes into account this random effect when comparing the different provinces, the different seasons and the SCC category as well as the interactions. Under the "goal of parsimony", a stepwise approach based on the smallest Akaike Information Criterion, was used to select the best model.

Abbreviations

AC: amox/k clav; AM: ampicillin; AZ: azithromycin; CE: cefepime; Class: number of antimicrobial classes that each isolate is resistant to; CLSI: clinical laboratory standards institute; CT: cefotaxime; CX: cefuroxime; CP: ciprofloxacin; DA: clindamycin, ET: ertapenem; E: erythromycin; FA: fusidic acid; FO: fosfomycin; FX: cefoxitin screen; G: gentamycin; GLMM: general linear mixed model; I: intermediate; IM: imipenem; LZ: linezolid; MDR: multi drug resistant; ME: meropenem; MIC: minimum inhibitory concentrations; MRSA: methicillin resistant *Staphylococcus aureus*; MU: muriprocin; N/A: not applicable; NS: not significant; (n): number of isolates; OX: oxacillin; PBP: penicillin binding protein; P: penicillin; R: resistance; RI: rifampin; SCC: somatic cell count; S; susceptible; SY: synercid; TEI: teicoplanin; TE: tetracycline; TO: tobramycin; T: total number of antimicrobial products resistant; VA: vancomycin

Declarations

Ethics approval and consent to participate

The study was a retrospective analysis approved by the University of Pretoria Ethics Committee (reference number V062/14). The laboratory that supplied the data provided written consent from owners for the data to be used for research purposes and this was approved by the ethics committee.

Consent for publication

The study does not involve human subjects and therefore no consent was required. However, the laboratory that supplied the study data provided consent for study results to be published.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

J.K. was the project leader, conceptualised and wrote the article and assisted in data collection and performed MIC testing. E.M.C.E. performed all the statistical analysis and

assisted in writing up the portions related to the analysis. E.F.D., V.N. and I.-M.P. assisted in conceptualising and writing the article as well as reviewing the manuscript. E.F.D. also assisted with language editing. I.-M.P. assisted with all udder health-related portions and V.N. assisted with the pharmacology and MIC -related aspects.

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Tables

Table 1 Summary of differences in antimicrobial resistance (GLMM) according to CLSI [6; 7], previous system.

Antimicrobial	Year	Season	Province	SCC Category
Tylosin	P < 0.01*	P=0.08 Autumn(Highest R) & Spring (Lowest R) *	NS	NS*
Penicillin G	P = 0.04*	P<0.01 Autumn (highest R)& Spring (Lowest R)*	NS	NS*
Ampicillin	P = 0.02*	P=0.02 Autumn (highest R)& Spring (Lowest R)*	NS	NS*
Clindamycin	N/A	N/A	N/A	N/A
Cefuroxime[a]	NS	NS	NS	NS*
Cefalonium (2011-2017)	P = 0.08*	NS	NS	P= 0.01 Med SCC (Highest R)& High SCC (Lowest R)*
Cefoxitin (2014-2017)	NS	NS	NS	NS*
Oxy-tetracycline	P = 0.003*	NS	NS	NS*
Cephalexin[b]	P = 0.007*	P=0.07 Summer(Highest R)&Autumn(Lowest R)*	NS	NS
Cloxacillin	NS	NS	NS	P<0.05 Low SCC (Highest R) & High SCC (Lowest R)*

See text for detailed explanation

*The variable/s included in the best statistical model (GLMM), even if NS.

NS = Not Significant, R = Resistance, N/A = Not applicable

Table 2 Summary of differences in antimicrobial resistance (GLMM) according to CLSI [8; 9], more recent system.

Antimicrobial	Year	Season	Province	SCC Category
Tylosin	NS	NS	NS	NS*
Penicillin G	P = 0.06*	P=0.0011 Autumn & P = 0.0019 Summer (Highest R) & Spring (Lowest R)*	NS	NS*
Ampicillin	P = 0.031*	P=0.000374 Autumn & P = 0.042 Summer (Highest R) & Spring (Lowest R)*	NS	NS*
Clindamycin	N/A	N/A	N/A	N/A
Cefuroxime[a]	NS	NS	NS	NS*
Cefalonium (2011-2017)	NS	NS	NS	NS
Cefoxitin (2014-2017)	NS	NS*	NS	NS*
Oxy-tetracycline	NS	NS	NS	NS*
Cephalexin[b]	P = 0.008*	P=0.0 12 Summer (Highest R) & Winter (Lowest R)*	NS	NS*
Cloxacillin	NS	P=0.0 58 Summer (Highest R) & Autumn (Lowest R)*	NS	P = 0.067 Low SCC (Highest R) & High SCC (Lowest R)*

See text for detailed explanation

*The variable/s included in order to achieve the best statistical model (GLMM), even if NS.

NS = Not Significant, R = Resistance, N/A = Not applicable

Table 3 Distribution of MIC cumulative percentage for maltose positive and maltose negative *S. aureus.*

Product	% Resistance	STA (+) (n = 57)												% Resistance	STA (-) (n = 57)						
		Distribution of MICs % (µg/ml)													Distribution of MICs % (µg/ml						
		0.03	0.06	0.12	0.25	0.5	1	2	4	8	32	64	256		0.03	0.06	0.12	0.25	0.5	1	2
Amox/K Clav	3.5	a	a	a	a	89[a]*	99[a]*	99[a]*	99[a]*	99*				21.1	a	a	a	a	77[a]*	89[a]*	91[a]*
Ampicillin		a	a	a	a	70*	78*	85*	90*	90*					a	a	a		56*	63*	68*
Azithromycin	3.5	a	a	a	a	a	98	98		b	b	b	b	1.8	a	a	a	a		98*	98*
Cefepime	1.8	a	a	a	a	a	a	a	100[a]	100[a]	b	b	b	22.8	a	a	a	a	a	a	a
Cefotaxime	1.8	a	a	a	a	a	93[a]*	100[a]*	a	a	b	b	b	22.8	a	a	a	a	a	88[a]*	96[a]*
Cefoxitin	1.8	a	a	a	a	a	a	a	100*					0	a	a	a	a	a	a	
Cefuroxime	1.8	a	a	a	a	a	a	a	100*	100*				21.1	a	a	a	a	a	a	
Ciprofloxacin	7	a	a	a	a	89*	94*							1.8	a	a	a		95*	98*	
Clindamycin	5.3	a	a	a	91[a]*	96[a]*	_*	98*	b	b	b	b	b	24.6	a	a	a	68[a]*	77*	_*	86*
Daptomycin[#]	0	a	a	a	a	95[a]*	100[a]*	100*	100*					17.5	a	a	a	a	82[a]*	82*	82*
Ertapenem[#]	3.5	a	a	a	a	98[*a]	98[a]*				100			1.8	a	a	a	a	97[a]*	97*	
Erythromycin	1.8	a	a	a	a	a	99*	99*		b	b	b	b	15.8	a	a	a	a		82*	84*
Fosfomycin[#]	1.8	a	a	a	a	a	a	a	a	a	98[a]*	a	b	12.3	a	a	a	a	a	a	a
Fusidic Acid[a]	0							100*						10.5							89*
Gentamicin	7	a	a	a	a	a	93[a]*	98[a]*	100[a]*		b	b	b	22.8	a	a	a	a	a	79[a]*	98[a]*
Imipenem	3.5	a	a	a	a	a	a	99[a]	99[a]	99	b	b	b	21.1	a	a	a	a	a	a	100[a]*
Levofloxacin[#]	0	a	a	a	a	a	100[a]*	100[a]*		b	b	b	b	0	a	a	a	a	a	100[a]*	100*
Linezolid[#]	0	a	a	a	a	21[a]*	64[a]	100[a]*	100*					15.8	a	a	a	a	21[a]*	37[a]*	82*
Meropenem[#]	1.8	a	a	a	a	a		100[a]*	100*	100*				21.1	a	a	a	a			100*
Moxifloxacin[#]	0	a	a	a	a	100[a]*	100*	b	b	b	b	b	b	0	a	a	a	a	100*	100*	b
Nitrofuratoin[#]	0	a	a	a	a	a	a	a	a	a	a	100*	b	0	a	a	a	a	a	a	a
Oxacillin	1.8	a	a	a	54[a]*	91[a]*	98[a]*	100[a]*						21.1	a	a	a	56[a]*	79[a]*	88[a]*	88*
Penicillin	36.8	61*	70*	70[a]*	70[a]*	_	_	76*						47.4	33*	40[a]*	53[a]*	54[a]*	_*	_*	63*
Rifampin[#]	0	a	a	a	a	100[a]*	100[a]*	100*	b	b	b	b	b	5.3	a	a	a	a	93[a]*	95*	95*
Synercid	5.3	a	a	a	a	a	100[a]*	100*	100[b]*	b	b	b	b	19.3	a	a	a	a	a	75*	77*
Teicoplanin[#]	0	a	a	a	a	a	100[a]*	100[a]*	100[a]*	100[a]*	b	b	b	14	a	a	a	a	a	82[a]*	84[a]*
Tetracycline	7	a	a	a	a	a	92[a]*	92[a]*	a					7	a	a	a	a	a	95[a]*	95[a]*
Tobramycin[#]	3.5	a	a	a	a	a	98[a]*	98[a]*	100[a]*		b	b	b	7	a	a	a	a	a	93[a]*	98[a]*
Trimeth/Sulfa	0	a	a	a	a	a	100*	100*	100*					0	a	a	a	a		98*	98*
Vancomycin	0	a	a	a	0[a]*	27[a]*	86[a]*	100[a]*	100*	100*	b	b	b	31.6	a	a	a	7[a]*	18[a]*	54[a]*	67*

CLSI Human breakpoints M100-S25 used, **antimicrobials with no superscript CLSI Veterinary breakpoints M31-A3used, * dilution ranges PM 32,**

[a] Denote the susceptible MIC, [b] Denote resistant MIC, for antimicrobials with only [a] the unmarked fields are taken as resistant, for antimicrobials with [a,] and [b] the unmarked fields in between denote Intermediate [8; 9].

Trimeth/Sulpha = Trimethoprim / Sulphamethoxazole; STA + = maltose positive *S. aureus*; STA - = maltose negative *S. aureus*; n = number of isolates

Table 4 MIC results [8; 9] of maltose positive and maltose negative *S. aureus* resistant >1 antimicrobial.

Isolate		(n)	Products																										
	T	Class	AC	AM	AZ	CE	CT	FX	CX	CP	DA	DP	ET	E	FX	FA	G	IM	LZ	ME	MU	OX	P	RI	SY	TEI	TE	TO	VA
STA-	2	1	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	S	S*	S	S*	S	S*	R	S	S	S	S	S	S
STA+	2	1	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
STA-	2	1	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
STA-	2	1	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA-	2	1	S	R	S	S	S	S	S	S	I	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA-	2	1	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
STA-	3	3	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
STA-	3	2	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	R	S*	S	S*	S	S*	R	S	S	S	S	S	S
STA-	3	2	S*	R	S	S*	S*	S	S*	R	S	S	N/A	S	S	S	S	S*	S	S*	S	S*	R	S	S	S	S	S	S
STA-	3	2	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S
STA+	3	2	S	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
STA+	3	2	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	S
STA-	4	3	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	R
STA-	3	3	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R
STA-	3	3	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
STA-	3	3	S	R	S	S	S	S	S	S	R*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA+	4	3	S	R	R	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA-	4	3	S	R	S	S	S	S	S	S	I	S	S	S	S	S	R	S	S	S	S	S	R	S	I	S	S	S	R
STA-	4	3	S	R	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
STA-	5	3	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	R	S*	S	S*	S	S*	R	S	S	S	S	R	R
STA+	5	3	S	R	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S	S	S	S	R	S	S	S	S	R	S
STA+	5	2	R	R	S	S	S	S	S	S	I	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S	S	S
STA-	5	3	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R	R	S	S	S	R
STA-	5	3	S	R	S	R	R	S	S	S	R	S	S	S	R	S	S	S	S	S	N/A	S	S	S	S	S	S	S	S
STA-	4	3	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	R	S	S	S*	S	S*	S	S*	R	R	S	S	S	S	S
STA-	5	3	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	R	S*	S	S*	S	S*	R	S	S	S	S	R	R
STA-	6	3	S	R	S	S	S	S	S	S	R	R	S	R	S	S	S	S	R	S	S	S	S	S	R	S	S	S	S
STA-	9	3	R	R	S	R	R	S	R	S	S	S	N/A	S	S	S	S	R	S	R	S	R	R	S	S	S	S	S	S
STA-	12	3	S	R	S	S	S	S	S	S	R	R	R	R	R	S	S	S	R	S	R	S	R	S	R	R	S	S	R
STA-	14	3	R	R	S	R	R	S	R	S	I	R	N/A	S	S	S	S	R	R	R	S	R	R	S	R	S	S	S	R
STA+	16	3	R*	R*	R	R*	R*	R	R*	S	R	S	R*	S	S	S	R	R*	S	R*	N/A	R	R*	S	R	S	S	R	S
STA-	16	3	R	R	S	R	R	S	R	S	R	R	N/A	I	S	R	S	R	R	R	N/A	R	R	S	R	R	S	S	R
STA-	18	3	R	R	S	R	R	S	R	S	R	R	N/A	R	S	R	S	R	R	R	R	R	R	S	R	R	S	S	R
STA-	20	3	R	R	R	R	R	S	R	S	R	R	N/A	R	S	R	S	R	R	R	N/A	R	R	R	R	R	R	S	R
STA-	21	3	R	R	S	R	R	S	R	S	R	R	N/A	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	R
STA-	20	3	R	R	S	R	R	S	R	S	R	R	N/A	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R
STA-	21	3	R	R	S	R	R	S	R	S	R	R	N/A	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R

T = Total number of antimicrobial products resistant, Class = Number of antimicrobial classes that each isolate is resistant to, S = Susceptible, R = Resistant, I = Intermediate, N/A = Not applicable, (n) = number of isolates

AC = Amox/K Clav, AM = Ampicillin, AZ = Azithromycin, CE = Cefepime, CT = Cefotaxime, FX = Cefoxitin Screen, CX = Cefuroxime, CP = Ciprofloxacin, DA = Clindamycin, ET = Ertapenem, E = Erythromycin, FO = Fosfomycin, FA = Fusidic Acid, G = Gentamycin, IM = Imipenem, LZ = Linezolid, ME = Meropenem, MU = Muriprocin, OX = Oxacillin, P = Penicillin, RI = Rifampin, SY = Synercid (Quinupristin-Daldopristin), TEI = Teicoplanin, TE = Tetracycline, TO = Tobramycin, VA = Vancomycin

Figures

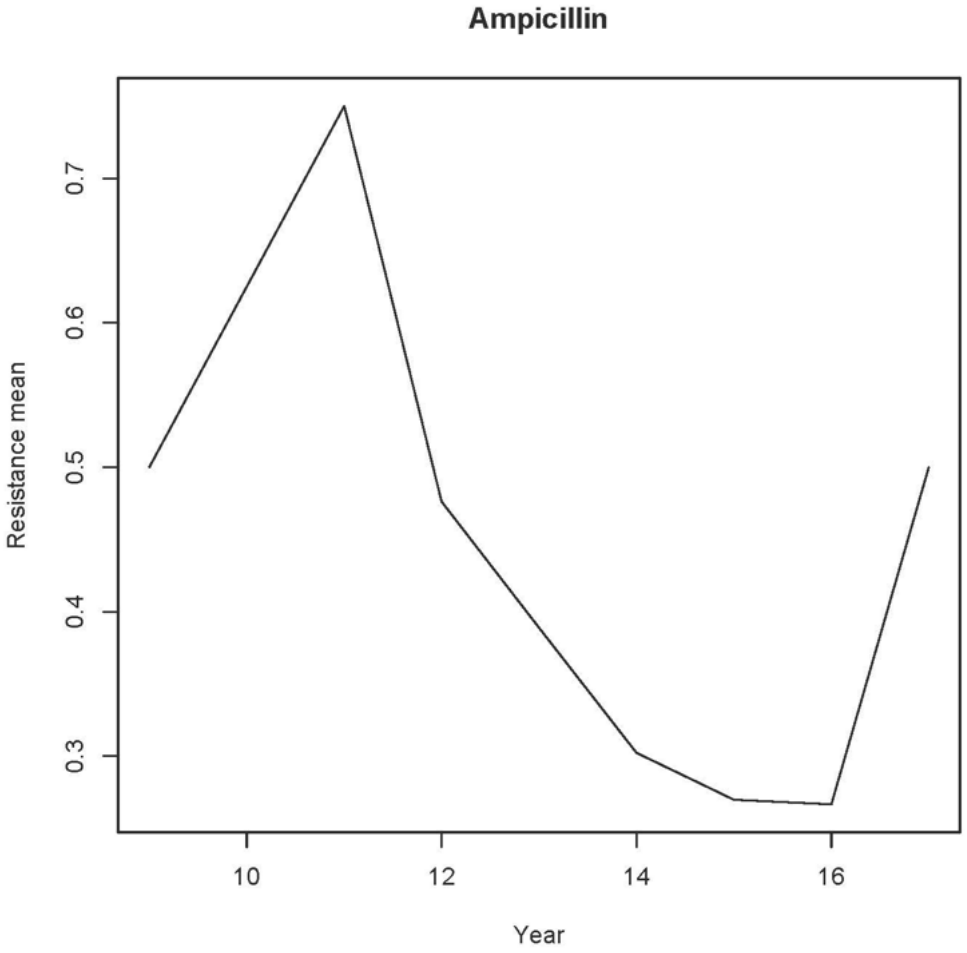

Figure 1

Trends of antimicrobial resistance to ampicillin over time of maltose negative S. aureus. Resistance mean is the proportion of isolates per year; this figure used the disc diffusion, retrospective data, according to CLSI [6; 7], with intermediate responses grouped with resistant responses (the previous system).

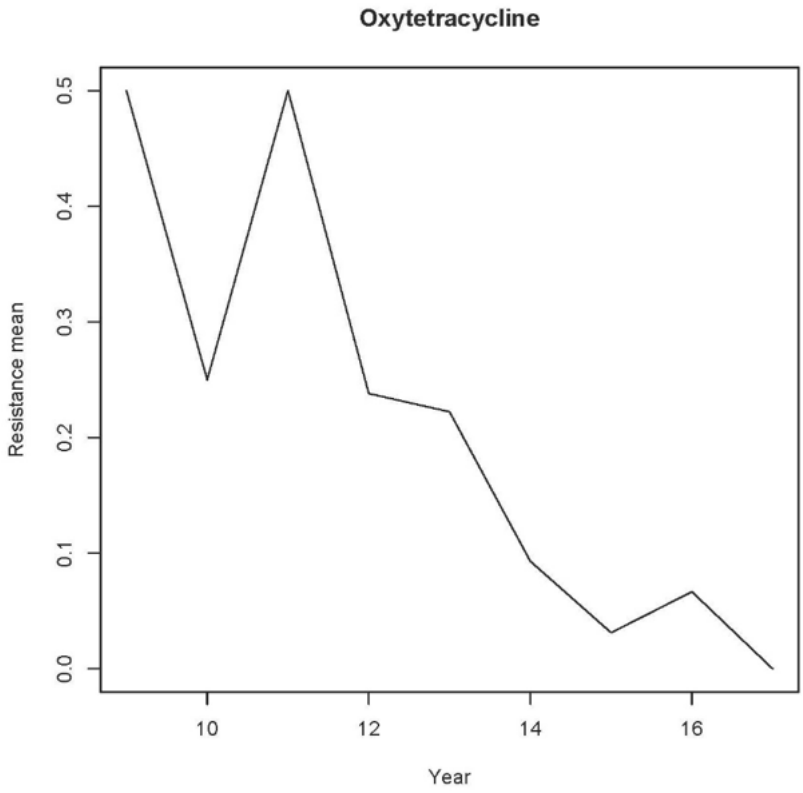

Figure 2

Trends of antimicrobial resistance over time to oxy-tetracycline of maltose negative S. aureus. Resistance mean is the proportion of isolates per year; this figure used the disc diffusion, retrospective data, according to CLSI [6; 7], with intermediate responses grouped with resistant responses (the previous system).

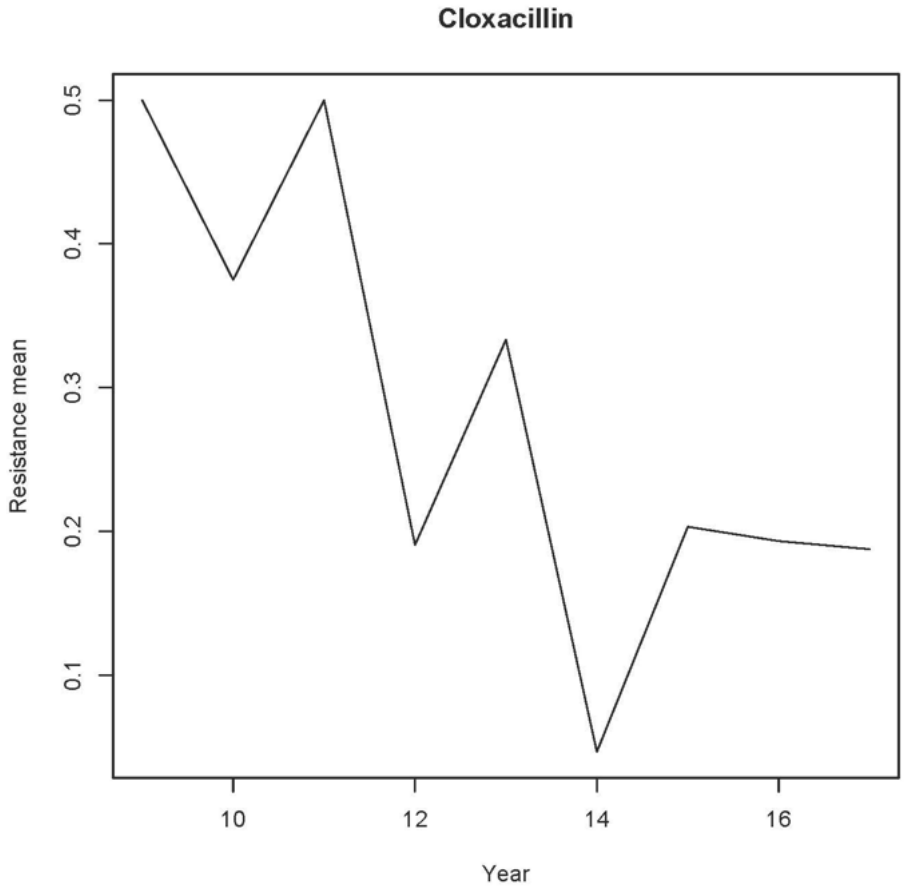

Figure 3

Trends of antimicrobial resistance to cloxacillin over time of maltose negative S. aureus. Resistance mean is the proportion of isolates per year; this figure used the disc diffusion, retrospective data, according to CLSI [6; 7], with intermediate responses grouped with resistant responses (the previous system).

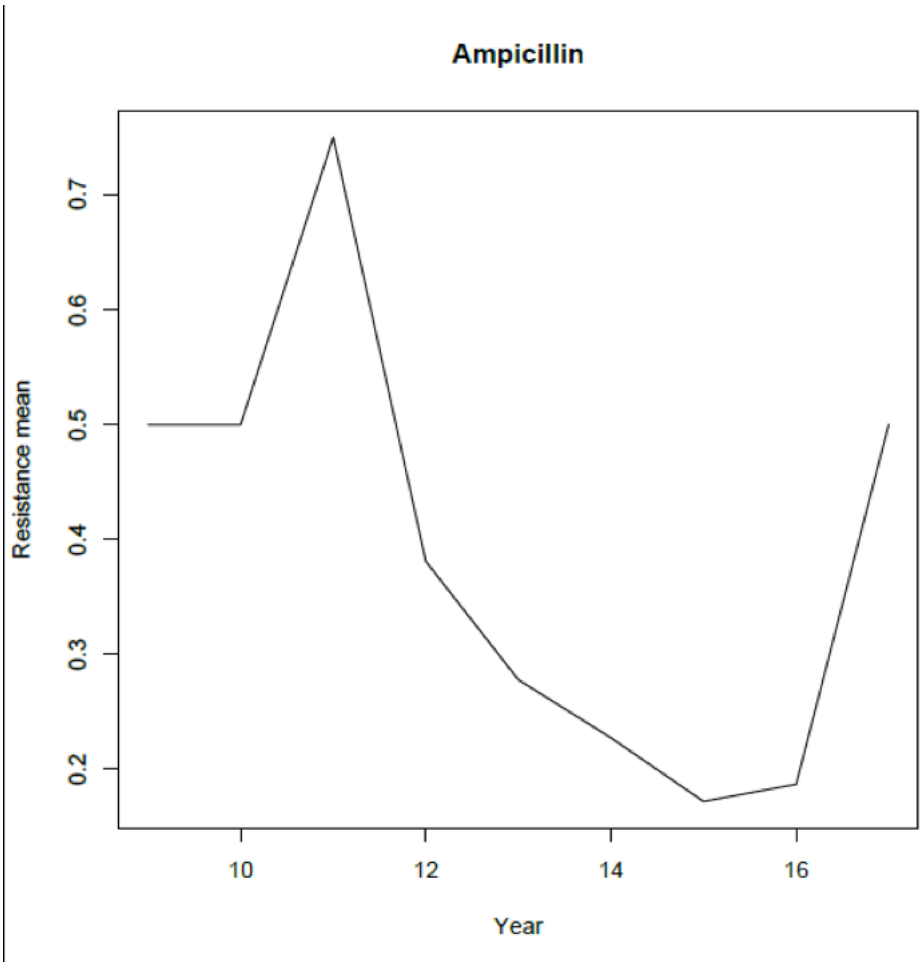

Figure 4

Trends of antimicrobial resistance to ampicillin over time of maltose negative S. aureus. Resistance mean is the proportion of isolates per year; this figure used the disc diffusion, retrospective data, according to CLSI [8; 9], with intermediate responses grouped with susceptible responses (the more recent system).

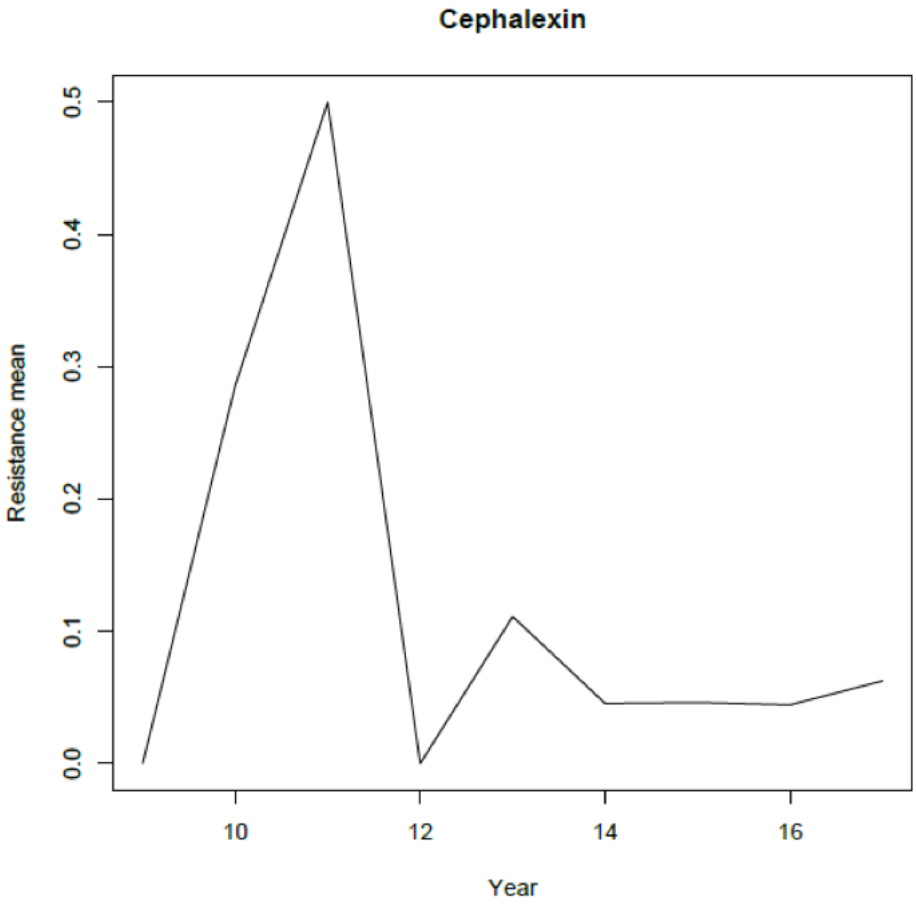

Figure 5

Trends of antimicrobial resistance to cephalexin over time of maltose negative S. aureus Resistance mean is the proportion of isolates per year; this figure used the disc diffusion, retrospective data, according to CLSI [8; 9], with intermediate responses grouped with susceptible responses (the more recent system).

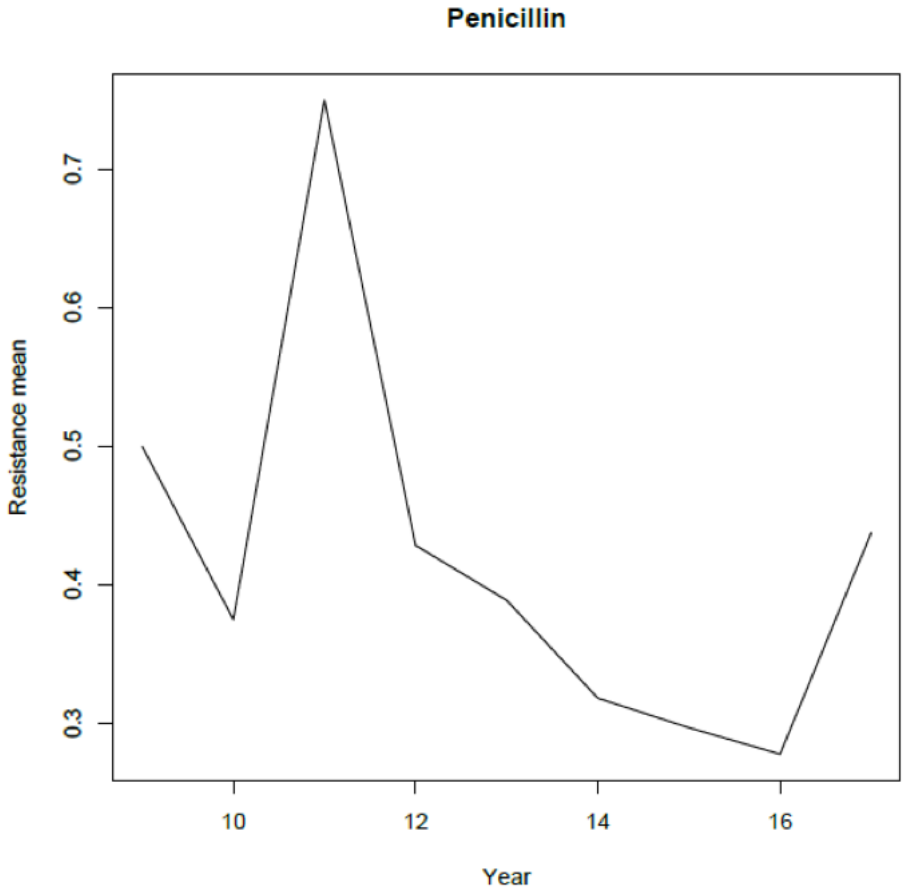

Figure 6

Trends of antimicrobial resistance to penicillin G over time of maltose negative S. aureus. Resistance mean is the proportion of isolates per year; this figure used the disc diffusion, retrospective data, according to CLSI [8; 9], with intermediate responses grouped with susceptible responses (the more recent system).