ANTIMICROBIAL RESISTANCE PATTERNS AND PLASMID PROFILES OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MILK AND MEAT

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ABSTRACT

Objectives: **To determine the frequency of resistance of *Staphylococcus aureus* to various antimicrobial agents, and the relationship between antimicrobial resistance of the isolates and carriage of plasmids.**
Design: **A random sampling of milk and meat samples was carried out.**
Setting: **Milk was collected from various dairy co-operative societies in Nairobi and Kiambu districts. Minced meat samples were purchased from various outlets in the city of Nairobi.**
Subjects: **Ninety six *Staphylococcus aureus* isolates from milk (seventy five isolates) and minced meat (twenty one isolates) samples.**
Main outcome measures: **Plasmid profiles and antimicrobial susceptibility tests to ampicillin, lincomycin, penicillin, erythromycin, methicillin, minocycline, cotrimoxazole and chloramphenicol.**
Results: **Seventy one per cent of the isolates carried between one and six plasmids of molecular sizes ranging from 0.1 to 14.5 kilobases. High frequency of resistance was observed with lincomycin (67.7%), penicillin (66.7%) and cotrimoxazole (51%). A high percentage (76%) of isolates were susceptible to minocycline followed by erythromycin (57.3%). Most (80.2%) of isolates were multiply resistant to between two and six antibiotics.**
Conclusion: **Most *Staphylococcus aureus* isolates were multiply resistant to various antimicrobial agents, but there was no apparent relationship between carriage of plasmids and antimicrobial resistance. Milk and meat may contain resistant *Staphylococcus aureus* posing a potential risk to consumers.**

INTRODUCTION

Staphylococcus aureus (S. aureus) is an important pathogen that causes skin, wound and burn infections, septicaemia and endocarditis, such that infections involving antibiotic resistant strains may impact on human health. Hospital associated (nosocomial) staphylococcal infections have been reported to be resistant to as many as 20 antimicrobial compounds, including antiseptics and disinfectants(1). Resistance to penicillin among *S. aureus* strains appeared a few years after the introduction of penicillin therapy(1,2). Introduction of other antibiotics such as streptomycin, tetracycline and chloramphenicol, and the macrolides was similarly followed by emergence of resistant organisms(2,3). Resistant organisms that had acquired resistance to these antibiotics were reported to be usually resistant to penicillin through the production of penicillinase(1). This resulted in the creation of organisms with a wide spectrum of resistance and a marked ability to survive and spread in the hospital environment(4). Such multiply resistant *S. aureus* strains were of global significance as early as 1950s(3,5). The introduction of synthetic beta lactamase resistant penicillins such as methicillin and oxacillin brought about a general decline in the prevalence of multiply resistant *S. aureus* during the early 1960s(3). By late 1960s and early 1970s, strains resistant to synthetic beta lactams were isolated with increasing frequency in a number of countries(1). Most methicillin resistant strains isolated at this time produced a beta lactamase and were also resistant to streptomycin, sulphonamides and tetracyclines, while many demonstrated additional resistance to chloramphenicol, erythromycin and fusidic acid or to the aminoglycoside, neomycin(6). Resistance to neomycin, and to the related aminoglycosides kanamycin and paromomycin, was attributed to the widespread topical use of neomycin on the skin and in the nose (7,8). During late 1970s and early 1980s, strains of *S. aureus* resistant to multiple antibiotics including methicillin and gentamicin were, increasingly responsible for outbreaks of hospital infections in countries around the world(1). Most of these outbreaks were associated with individual wards or units, with neonatal, intensive care and burns units being particularly susceptible(1,9,10). In some instances however, the persistence and propensity for spread of these organisms posed a much more serious

clinical problem involving numerous patients at several different hospitals(11,12). The organisms involved often possessed the capacity to counter almost all the antimicrobial agents available to the clinician for the treatment of severe staphylococcal infections(1). Other antibiotics that have been used for the clinical treatment of *S. aureus* infections include fusidic acid, rifampicin and vancomycin(1,13)

Resistance to the first two agents was reported much earlier than the report of resistance to vancomycin. Vancomycin remained the drug of choice for methicillin and bata lactam resistant staphylococci(14,15), but recently, vancomycin or other glycopeptide intermediately resistant *S. aureus* (VISA/GISA) have emerged. Risk factors for VISA/GISA are less well understood although both organisms emerge in patients receiving vancomycin or other glycopeptide antibiotics.

The appearance of antibiotic-resistant staphylococci over the years was regarded as an inevitable genetic response to the selective pressure imposed by antimicrobial use(1). Resistance to antimicrobial agents in bacteria may occur due to a spontaneous mutation(16), or may be acquired through transmission from other resistant bacteria(17). Plasmids may contain resistance genes for single or multiple antimicrobial agents and they have been reported to transfer these resistance from one bacteria to another(17,18). Indeed, resistance within the staphylococci to several therapeutically useful antibiotics, including streptomycin, rifampicin, fusidic acid and novobiocin is thought to be derived from chromosomal mutation(19). In many instances, resistance to antimicrobial agents in staphylococci has also been due to plasmids that carry the genetic determinants of resistance(6,20). Plasmid profiles have been found useful in epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance(21,22) The aim of this study was to determine the frequency of resistance of *S. aureus* isolates from milk and meat to various antimicrobial agents, screen the isolates for carriage of plasmids, and determine the relationship (if any) between antimicrobial resistance of the strains and carriage of plasmids.

MATERIALS AND METHODS

Bacterial strains: A total of ninety six *S. aureus* strains isolated from milk (75 strains) and meat (21 strains) were used. *Staphylococcus aureus* strain ATCC 25923 was used as reference.

Test for antibiotic susceptibility: Staphylococcus aureus isolates were tested for their susceptibility to eight different antimicrobial agents by use of disc diffusion technique(23). Bacterial strains were inoculated into Mueller Hinton broth (Oxoid, Unipath, United Kingdom) and incubated at 37°C for 18-24 hours. The inoculum was standardised by adjusting the turbidity of an actively growing broth culture to match that of a barium sulphate (0.5 Macfarland) standard. Mueller Hinton agar plates (Oxoid, Unipath, United Kingdom) were then inoculated with this bacterial suspension and spread as evenly as possible. One KGL 1/4 multodisk (Lab M, England) containing eight different antimicrobial agents namely: ampicillin (10 μg), lincomycin (2 μg), penicillin (10 i.u), minocycline (30 μg), erythromycin (15 μg), methicillin (5 μg), cotrimoxazole (25 μg)

and chloramphenicol (30 μg) was then placed on each of the inoculated Mueller Hinton agar plates and incubated at 37°C for 18-24 hours. After incubation, the diameter of the inhibition zone for each antibiotic was measured to the nearest millimetre. Isolates were described either as resistant (not inhibited), inter-mediately resistant (not completely inhibited), or sensitive (appropriately inhibited, with the recommended dosage of a particular antibiotic), based on the size of inhibition zones(23). A Chi-square test was used to compare the difference between the proportions of the isolates from milk and meat that were resistant to various antimicrobial agents.

Plasmid profile analysis: Plasmid DNA was isolated as described by Birnboim and Doly(24) modified by use of lysostaphin for lysing the cell wall. Each *S. aureus* strain was inoculated into 3 ml tryptic soy broth and incubated overnight on a roller drum at 37°C. About 1.5 ml of each overnight broth culture was transferred into eppendorf tubes and centrifuged for one minute at 15000 rev/min at room temperature. The supernatant was discarded and 2 μl of lysotaphin solution (1.0 μg/ml in distilled water) added to the pellet. Tubes were capped, vortexed and placed in ice for 30 minutes. Two hundred microlitres of alkaline detergent solution (0.2 N NaOH; 1% SDS) was added and tubes inverted several times and then kept in the waterbath for five minutes. One hundred and fifty microlitres of 3 M Sodium acetate (pH 4.8) was added and tubes inverted several times to mix and then kept in ice for at least 10 minutes. The tubes were centrifuged at room temperature at 15000 rev/min for five minutes and the supernatant transferred into new eppendorf tubes. One millilitre of 95% ice cold ethanol was added to the tubes, which were then kept at -20°C for five minutes. After five mins, they were centrifuged at 15000 rpm for three minutes, supernatant discarded and the sediment resuspended in 50 μl of Tris/ETDA (10 mM Tris HCl and 1 mM ETDA. pH 8.0). Thirty three microlitres of the contents were then loaded into wells of 1.0% agarose gels containing ethidium bromide. A 1.0 kb DNA ladder (MBI, Fermentas, Vilnius, Lithuania) was run side by side with test isolates as a molecular size marker. Electrophoresis was carried in Tris Acetate ETDA buffer containing ethidium bromide (20 ml of 50 X TAE and 6.0 μl of 10 μg/ml ethidium bromide per litre) at 30 mA (90 V) for four hours. Plasmids were viewed on a U/V transilluminator and photographs taken using a Leicaflex SL-camera. Films were exposed for 90 seconds and later developed. Plasmid sizes were estimated from a standard curve drawn of the molecular sizes of the 1.0 kb DNA ladder against their migration distance.

RESULTS

Resistance to antibiotics: The frequency of resistance to various antibiotics among *S. aureus* isolates is shown in Table 1. A high frequency of resistance was observed with lincomycin (67.7%), penicillin (66.7%) and cotrimoxazole (51%). The highest number of isolates were susceptible to minocycline (76%), followed by erythromycin (57.3%), chloramphenicol (38.5%) and methicillin (33.3%). Eighty per cent (77 of 96) of the isolates were multiply resistant to at least two antimicrobial agents. Only one isolate was susceptible to all the antimicrobial agents tested. A higher proportion (44.9%) of isolates from meat were resistant to antimicrobial agents compared to 36% of milk isolates (p = 0.0394). A higher proportion of isolates from meat were resistant to ampicillin (61.9%; p=0.0006), penicillin (90.5%; p = 0.0088) and methicillin (42.9%; p = 0.0001)

compared to milk isolates. On the other hand, a higher proportion of isolates from milk were resistant to cotrimoxazole (56%; p = 0.0263), compared to meat isolates. There was no significant difference in resistance to lincomycin, minocycline, erythromycin and chloramphenicol between the isolates from meat and from milk (Table 2).

Table 1

Frequency of antimicrobial susceptibility among ninety six Staphylococcus aureus isolates

Antimicrobial agent	No.(%) isolates (n=96)		
	Resistant	Intermediate	Susceptible
Ampicillin	30 (31.3)	51 (53.1)	15 (15.6)
Lincomycin	65 (67.7)	20 (20.8)	11 (11.5)
Penicillin	64 (66.7)	30 (31.3)	11 (2.1)
Minocycline	11 (11.5)	12 (12.5)	73 (76.0)
Erythromycin	14 (14.6)	27 (28.1)	55 (57.3)
Methicillin	15 (15.6)	49 (51.0)	32 (33.3)
Cotrimoxazole	48 (51.0)	20 (20.8)	28 (29.2)
Chloramphenicol	38 (39.6)	21 (21.9)	37 (38.5)

Table 2

Frequency of antimicrobial resistance among Staphylococcus aureus isolated from milk and meat

Antimicrobial agent	No (%) resistant isolates		
	Milk isolates n = 75	Meat isolates n = 21	p-value
Ampicillin	17 (22.7)	13 (61.9)	0.0006
Lincomycin	55 (73.3)	12 (57.0)	0.1532
Cotrimoxazole	42 (56.0)	6 (28.6)	0.0262
Penicillin	45 (60.0)	19 (90.5)	0.0088
Minocycline	9 (12.0)	2 (9.5)	0.7528
Erythromycin	10 (13.3)	4 (19.0)	0.5120
Chloramphenicol	32 (42.7)	6 (28.6)	0.2430
Methicillin	6 (8.0)	9 (42.9)	0.0001

Plasmid profiles: The *S. aureus* isolates were grouped into eight plasmid profile groups according to the number of plasmids they contained, with those that had no plasmid in one profile group. About seventy one per cent (70.8%) of all the isolates contained plasmids. The number of plasmids per isolate ranged between one and six, but most isolates contained between one and four plasmids (Table 3). A variety of plasmids were found with molecular sizes ranging from 0.1 to 14.5 kilobases. The most common plasmid sizes encountered were 1.0 and 0.7 kb. Other molecular size plasmids were found in a relatively low frequency.

Table 3

Plasmid profile groups of Staphylococcus aureus isolated from milk and meat

Plasmid profile group	No. of isolates	Plasmid sizes(Kb) represented
0	28	None
1	10	0.7, 1.0, 3.0, 6.0
2	34	0.7, 1.0, 1.5, 1.9, 3.5, 4.0, 12.0, 13.0
3	7	0.7, 1.0 1.4, 1.8 1.9, 2.5, 4.0, 5.0, 13.0, 14.5
4	15	0.7, 1.0, 1.4, 1.6, 1.8, 2.0 2.3, 2.5, 3.0, 3.5, 4.0, 6.0 10.0, 13.0, 14.5
5	1	0.7, 1.0, 1.3, 1.6, 5.0
6	1	0.7, 1.0, 1.3, 1.8, 2.0, 2.8

Relationship between plasmid profiles and antibiotic resistance patterns: There was no apparent relationship between carriage of plasmids and antibiotic resistance patterns as resistance to specific antibiotics was observed in isolates with various molecular weight plasmids as well as in those strains that had no plasmids.

DISCUSSION

This study revealed that milk and meat products may be contaminated with multiply resistant *S. aureus.* The high frequency of resistance observed with lincomycin (67.7%), penicillin (66.7%) and cotrimoxazole (51%) could be attributed to their use in treatment of diseases in animals and humans. Resistant bacteria may transfer resistance genes to other bacteria and become important in the spread of antibiotic resistance. Indiscriminate use of antimicrobial agents and antibiotic sale behaviour (for example, sale of antibiotics without prescription, sale of under dose and substituting brands) enhances the development of drug resistance(25).

Bacterial infection of human beings through consumption of contaminated food is a common finding worldwide. If care is not taken during milk processing or cooking of meat so as to destroy all the bacteria present in the products, resistant *S. aureus* in these products may find their way to the human body where they can cause disease that may prove difficult to treat. In developed countries, the main reservoirs for antimicrobial drug resistance in enteric bacteria has been attributed to farm animals such as cattle, sheep, pigs and poultry(21,26-30). Contact with these animals or consumption of food products from them has been the main route of dissemination of resistance into the human populations. Therefore, transmission of drug resistant bacteria from farms into the community and subsequently to patients in hospital may occur through food(21,28,29). This demonstrates how resistant bacteria arising from indiscriminate use of antibiotics in animals

may impact on human health. Isolation of the antibiotic resistant bacteria has been reported from pasteurised milk(30, 31).

Isolation of methicillin resistant *S. aureus* (MRSA) in this study was significant considering the problem these micro-organisms cause during nosocomial transmission. The problem of MRSA is worldwide. In the United States of America, MRSA represent 15-20% of all nososcomial *S. aureus* infections in tertiary care centres(32). The problem of MRSA has been reported to be common in both public and private hospitals in Kenya, where they seem to have taken permanent residence(33). These MRSA are known to be resistant to beta lactams including penicillins, cephalosporins, carbepenens, and disinfectants and toxic metals such as mercury. This study found 15.6% of *S. aureus* isolates to be resistant to methicillin, and all the MRSA isolates to be multiply resistant to a number of other antibiotics. Most (87%) of them were either resistant or intermediately resistant to ampicillin and penicillin. A higher percentage (42.8%) of isolates from minced meat were methicillin resistant, while only 8% were isolated from milk. This may be attributed to excessive handling of the meat with contaminated hands during the mincing process.

Plasmid profiles have been reported to be useful in tracing the epidemiology of antibiotic resistance(21,22). However, in this study, resistance to various antimicrobial agents was not associated with presence of plasmids. This was because no particular molecular size plasmid could be associated with any particular antimicrobial resistance. Resistance was observed in isolates with various molecular size plasmids as well as in those that had no plasmids. This could be attributed to the variety of sources of our milk and meat samples from where the *S. aureus* were isolated. Resistance of *S. aureus* isolates to various antimicrobial agents may be located either on chromosomes, plasmids or transposons. For example, methicillin resistance gene (mec4916) has a chromosomal locus, and is probably maintained on a mobile element(34,35), while tetracycline resistance observed in *S. aureus* strain DU4916 was reported to be encoded by a 4.0 kb plasmid(36). Noble and Rahman(37) reported tetracycline resistance in *S. aureus* to be encoded by a 4.3 kb plasmid, while in some strains resistance was found to be encoded by a chromosomal gene.

This study concluded that multiply resistant *S. aureus* and MRSA have a wide distribution in milk and meat products and care should be taken during processing of these products to destroy these micro-organisms to avoid the risk of human infection. In addition, there was no enough evidence to suggest that some particular plasmids carried by the isolates were responsible for resistance to any particular antibiotic(s).

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