Occurrence of Multiple Antimicrobial Resistance among *Staphylococcus aureus* Isolates from Kenyan Milk

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Abstract

Two hundred and sixteen isolates of *staphylococcus aureus* were obtained from raw milk within different locations in the Rift Valley of Kenya. Their resistance profiles to six families of antimicrobials were evaluated using the plate diffusion method. Resistance to penicillin (72.2 %) was the most frequent followed by trimethoprim + sulfamethazin (59.2 %); tetracycline (57.9 %); erythromycin (21.3 %); chloramphenicol (46.8 %) and methicillin (7.8 %). Multiple resistances, to penicillin and at least two other non - ß-lactam classes of antimicrobials, were observed in 76.9 % of isolates. Multiple resistances to more than four antimicrobials were 13.4 % while 1.9 % were susceptible (non resistant) to all six antmicrobials tested. Pearsons $\div^2$ statistic was determined to be 10.98 and $\div^2_{,6df} = 12.59$ (at P = 0.05), under the null hypothesis of no association for the five regions and resistance pattern. It was concluded that while most of the isolates (76.9 %) were multiple resistance to the tested antimicrobials, there was independence (at the 0.05 level of significance) between the sensitivity levels evident and the regions.

Key words. Milk; Staphylococcus aureus; Antimicrobial Resistance; Multiple resistance

Introduction

Antimicrobials used in animal husbandry for mastitic therapy purposes are often targerted at *Staph. aureus* as it is the pathogen most frequenly associated with bovine mastitis (Quinn *et al.* 1994). The question as to whether the use of antimicrobials, both therapeutics and growth promoters, leads to selection of resistant organisms has been studied since the 1950s (Scheidy *et al.* 1969). In several instances (Molitoris *et al.* 1986; Kaukas *et al.* 1988 and Niemi *et al.* 1993) a selective pressure has been shown to occur on the emergence and maintenance of drug resistance on the microbial flora of animals.

These studies have however mostly focussed on human clinical situations and there are fewer reports on the trends of antibiotic resistant bacterial strains in food in Africa. While *Staphylococcus aureus* poses potential food safety hazard (Bergdoll 1990), it is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Thus, the presence of this bacterium or its enterotoxins in processed foods or on processing equipment is generally an indication of poor sanitation.

In Kenya, many small and medium holder producers sell raw unpasteurized milk directly to consumers and this could be a likely entry route for resistant strains to humans. Improper pasteurized milk could also result in situations where milk is consumed with the bacteria. Evidence has been found which indicates that resistance strains of pathogens can be transmitted to humans through food (Oosterom 1991; Khachatourians 1998). The purpose of this study was to assess the occurrence extent of resistant *staph. aureus* isolates in raw milk, from four collection centres within the Rift Valley of Kenya .

Materials and Methods

Sample collection. Milk samples were randomly obtained from suppliers to four collection centres within the Rift Valley of Kenya between July 2001 and February 2002. The centres (Njoro/ Molo, Nyahururu, Nakuru and Naivasha) were all within a 100 Kilometre radius from the Egerton University Njoro. Sampling was asceptically done as per IDF standards 50 B (1995). The samples were transported cooled in ice boxes to the microbiology laboratory, department of Food science, Egerton University Njoro and stored at 4 °C. They were then examined within 24 hours.

Bacteria. Isolates of *Staph. aureus* were obtained from the milk samples according to established routine procedures (Arbeit 1998). Fifty four isolates were sampled from each of the regions (total = 216). Multiple isolates from the same milk suppliers were excluded. They were all identified on the basis of gram staining, colony morphology & haemolytic pattern on blood agar (Difco, Detroit, MI, USA) and confirmed by the the coagulase test (rabbit plasma in tubes) and other ancillary tests (catalase, anaerobic utilsation of mannitol and lysostaphin sensitivity) as per Arbeit (1998). Each of the isolates harvested was stored in a tube (Nalgene cryogenics vials) containing 1 ml of Trypticase soy broth and 15 % glycerol for prolonged storage at -31°C.

Susceptibility testing. Each *Staph. aureus* isolate was sub cultured on blood agar base, (Difco, Detroit, MI, USA) at 37 °C for 24 h before testing. They were then inoculated into Mueller Hinton broth (Oxoid; Amsterdam, The Netherlands) and incubated at 37 °C for

24 hours. The turbidity of the actively growing culture was adjusted to correspond with that of a barium sulphate (0.5 Macfarland) standard. Resistance of the isolates to six antimicrobials from different families was then determined by the plate diffusion method. In the technique, direct bacterial suspension inoculation was made on Mueller Hinton Agar (Oxoid; Amsterdam, The Netherlands) in the presence of sensitivity multi disks (Oxoid; Amsterdam, The Netherlands). The control strains were *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 29213.

The strains were tested using six antimicrobials (penicillin; trimethoprim + sulfamethazin; tetracycline; erythromycin; chloramphenicol and methicillin). Inhibition zone diameters were measured after 24 hr incubation at 37 ^{O}C and the interpretive breakpoints for resistance determined by comparing inhibition zones according to the National Committe for Clinical Laboratory standards (NCCLS, 1998) guidelines. The pearson's chi square (calculated on actual data) was used to test the hypothesis of no association for the categories of antmicrobial resistance and the four regions.

Results

The sensitivity profiles of the strains are summarized in t **Tables 1 - 3**. From **table 1**, the resistance pattern for both intermediate and resistant strains (R + I) have penicillin with the most frequent overall rate of, n = 193 (89.4%). This is followed in decreasing order by tetracycline, n = 178 (82.4%); trimethoprim + sulfamethazin, n = 174 (80.6%); chloramphenicol, n = 140 (64.8%); erythromycin, n = 83 (38.4%) and methicillin, n = 56 (25.9%).

From **table 2**, multiple resistance, defined as lack of susceptibility to penicillin and at least two other non - ß-lactam classes of antimicrobial drugs, was observed in 76.9 % of isolate. Multiple resistance to 4 to 6 antimicrobials was observed in 13.4% of the isolates while *9.7* % of the isolates were susceptible to all six antimicrobials tested.

[a]The number within parenthesis (fe) are the expected cell frequencies calculated based on a 4 X 3 contingency table model on the null hypothesis being true. The fe was calculated in every cell of the table and the test statistic determined which approximated the chi-squared distribution (**table 3**).

For significance at the 0.05 level, chi-square should be greater than or equal to 12.59. The distribution is not significant, as (P) is less than or equal to 0.10. It is concluded that there was no association between the regions and the sensitivity levels evident.

Discussion

Multiple resistance capable of regional dissemination can emerge as a result of antimicrobial selection pressure in either livestock or humans (Moller 1995; Levin et al. 1997). In this study no obvious clustering of multiple resistant strains was observed in the localities sampled. Thre was also no evidence of association between the development of multiple resistance and the regions at the 0.05 significance. The high incidence of antimicrobial multiple resitance was however evident (**Table 2**) and is suggestive of a selection pressure in force within the regions.

Table 1. Frequency of *S.aureus* isolates (n = 216) susceptible to selected antibiotics at different concentration levels determined by the plate diffusion method

Antimicrobial group	No (%) isolates (n = 216)			
	Disc Content (µg)	Resistant (R)	Intermediate(I)	Susceptible (S)
MACROLIDES				
Erythromycine	15	46(21.3%)	37(17.1%)	133(61.6%)
CHLORAMPHENICOLS				
Chloramphenicol	30	101(46.8%)	39(18.1%)	76(35.2%)
BETA – LACTAMS				
Penicillin G	10	156(72%)	37(17.1%)	23(10.6%)
Methicillin	5	17(7.8%)	39(18.1%)	160(74.1%)
TETRACYCLINES				
Tetracycline	30	125(57.9%)	53(24.5%)	38(17.6%)
SULFONAMIDES				
Trimethoprim/Sulfameth	5 + 30	128(59.2%)	46(21.3%)	42(19.4%)

Table 2. Frequency pattern of antimicrobial resistant *S.aureus* isolates from four regions in Kenya

Region	No. of (%) Isolates		
	Sensitive to all antibiotics	Resistant to 1-3 antibiotics	Resistant to 4-6 antibiotics
Nyahururu	4 (1.9 %)	41(19.0 %)	9(4.2 %)
Nakuru	3(1.4 %)	49(22.7 %)	2(1.0)
Naivasha	8 (3.7 %)	39 (18.1 %)	7(3.2)
Njoro/Molo	6(2.8 %)	37(17.1 %)	11(5.1)
Total	***21 (9.7 %)***	***166(76.9 %)***	***29(13.4 %)***

Table 3. Categorical data analysis of *S.aureus* isolate proportions arranged in a 4x3 table

Region	Observed f_o, Expected[a] (f_e)			
	Sensitive to all antibiotics	Resistant to 1-3 antibiotics	Resistant to 4-6 antibiotics	Totals[b]
Nyahururu	4 (5.25)	41(41.5)	9(7.25)	54
Nakuru	3(5.25)	49(41.5)	2(7.25)	54
Naivasha	8 (5.25)	39 (41.5)	7(7.25)	54
Njoro/Molo	6(5.25)	37(41.5)	11(7.25)	54
Total	*21*	*166*	*29*	*216*

Degree of freedom = 6; Chi-square = 10.98

Two conditions are needed for antibiotic resistance to develop in bacteria. First, the organism must come into contact with the antibiotic at levels below the strains Minimum Inhibitory Concentrations (MICs). Then, resistance against the agent must develop, along with a mechanism to transfer it to daughter organisms or directly to other members of the same species (Noble *et al.* 1992). An earlier study in Kenya (Shitandi & Sternesjö 2001) found beta-lactam antibiotics residues to be prevalent in milk. The high frequency of penicillin resistance occurrence in this study could be due to the constant contact between the *Staph. aureus* and beta -lactams, at levels below their MICs. The four antimicrobial drugs for which a considerable frequency of resistance was observed (Penicillin, 72.2 %; Trimethoprim + sulfamethazine, 59.2 % and Tetracycline, 57.9 %) are known to be extensively used in developing countries (Hart ad Kariuki 1998; Okeke *et al.* 1999). These inexpensive drugs are widely available from distributors and can be purchased easily from certain dealers without a prescription.

The resistance pattern observed in the study should be of concern as it raises food safety and ethical issues. Resistant strains are potential causes of infection but more importantly are also potential reservoirs of resistant genes that could be transfered to other pathogens. Ingestion of resistant microorganisms through food and water can result in selection of resistant strains in humans (Levin *et al.* 1997; Levy 1997). The methicillin resitance (R = 7.8 % and I = 18.1%) may be reflective of the increasing prevalence pattern of methicillinase resistance *S. aureus* (MRSA) worldwide (Kreiswirth *et al.* 1993; Maslow *et al.* 1993 and Ayliffe 1997). MRSA are important due to their pathogenicity, limited treatment options and are transmissible. Their transmissibility would be a particular problem in a milk/food processing plant as personnel colonized with MRSA strains (carriers of the organism with no symptoms of infection), can spread them through contact with others. Hand washing and screening workers for MRSA should be performed to decrease transmission and reduce the number of infected workers with MRSA.

Apart from erythromycin and methicillin, other groups of antibiotics were observed to be ineffective against the isolates of *S. aureus*. This suggests that no single drug is fully effective against the organism and it would be desirable if milk processors ensured adequate pasteurization to eliminate potential pathogens. However irrespective of the processing conditions, the misuse of antibiotics at the farm level is the heart of the resistance problem. A multidisciplinary approach is thus required to tackle the emerged problem of resistance in Kenya and reinforce milk safety efforts.

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