Antimicrobial Resistance Pattern of Streptococci and Staphylococci Isolated from Cases Of Bovine Clinical Mastitis in Nigeria.

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Abstract: Streptococci and staphylococci are frequently isolated from bovine mastitis in dairy cows. Limited information is available on the antimicrobial susceptibility of these organisms in Nigeria. A total of 130 Streptococci and 177 Staphylococci isolated from cases of bovine mastitis from three states in Nigeria namely; Oyo, Kwara and Kaduna states for a period of one year were used in this study. Overall, 55.38% of the strains tested were Streptococcus uberis, 24.62% were Streptococcus agalactiae, 12.31% were Streptococcus dysgalactiae, 3.85% were Streptococcus zooepidemicus, 2.31% were Streptococcus bovis and 1.54% were for Streptococcus equines, 25 coagulase negative staphylococcus and 152 Staphylococci aureus. The antimicrobial susceptibility for these organisms was determined for the following antimicrobial agents: Ampicillin, Tetracycline, Sulphadimidine, Nalidixic acid, Neomycin and Streptomycin. Results demonstrated substantial differences in their resistance patterns for the various organisms. The resistance patterns revealed 10 distinct resistance groups. All the streptococci isolate showed resistance to Ampicillin and tetracycline while 98.46%, 86.15%, 48.46%, 24.62%, and of the Streptoccoci species were resistance to sulphadimidine, Neomycin, Streptomycin and Nalidixic acid respectively while Staphyloccocus aureus had 100% for tetracyclin and sulphadimidine respectively while 97.37%, 88.16%, 69.08% and 67.11% showed resistance to ampicillin, neomycin, nalidixic acid and streptomycin respectively. However, Staphylococcus coagulase negative showed 100% resistance to tetracycline and sulphadimidine while 72%, 68%, 68%, and 32% showed resistance to nalidixic acid, neomycin, ampicillin and streptomycin respectively. The result of this study revealed that dairy farmers misused these antibiotics by treating cows several times per case. This study point to the fact that dairy farmers should take caution in the use of antibiotics for the dairy cows on the farms.
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1. Introduction

Mastitis is the most frequent and most expensive disease of dairy cows (Anaya-Lopez *et al*., 2006). *Streptococci s*pecies and *Staphylococcus aureus* are the major cause of infectious diseases to human and animal. *Streptococci species* and *Staphylococcus aureus* are predominant etiological agents of both subclinical and clinical forms of udder inflammation leading to great economic losses in the dairy industry (Osteras, 2005; Barkema *et al*., 2006). The role of coagulase-negative staphylococcus (CNS) has increased in recent as major causes of subclinical mastitis (Khan *et al.*, 2003).

Members of the family streptococcaceae have long been recognized as causative agents of bovine mastitis (Garcia, 2004). *Streptococcus agalactiae, S. dysgalactiae* and *S. uberis* have been reported as the three most commonly isolated species (Keefe, 1997.). Other *streptococcal species* have been implicated in bovine mastitis such as *Streptococcus bovis, Streptococcus zooepidemicus* and *Streptococcus equinus* although their relative frequency appeared low (Garcia, 2004). *Streptococcus pyogenes* intramammary infections have been associated with close contact of infected humans with susceptible cows (Barkema *et al*., 2006) the milker or attendant can contaminate the udder with *Streptococcus pyogenes* during milking. Epidemics of scarlet fever and septic sore throat as a result of consuming raw milk from *S. pyogenes* infected cows have also been reported (Barkema *et al.*, 2006).

Streptococcus agalactiae is a common mastitis agent whose eradication from individual herd is practical and cost effective. Eradication of *S. agalactiae* intramammary infections in dairy herds became feasible with the introduction of antibiotics and the advent of effective mastitis control procedures (Erskine *et al*., 2002). However, *S. agalactiae* remains a problem in individual herds (Bradley, 2002) and in countries lacking organized mastitis control programs (Myllys and Rautala 1995). It has been observed that mastitis caused by *Streptococcus agalactiae* should be suspected in a herd if cow or bulk tank somatic cell count (SCC) begins to rise and remains high, especially when bulk milk SCC is 1,000,000 cells/ml or higher (Djabri *et al*., 2002). An occasionally high bacteria count in bulk milk in infected udders is associated with high numbers of

Streptococcus agalactiae in the milk (Djabri *et al*., 2002).

Streptococcus agalactiae primarily infects the cisterns and the ductile system of the mammary gland. An irritation is produced causing inflammation of the gland which is mostly subclinical with occasional clinical symptoms (Ekin and Gurturk 2006.). Accumulation of bacteria waste products intensifies the inflammatory response resulting in destruction of milk producing tissue and reduced milk yield or produce agalactia. *Streptococcus agalactiae* rarely causes illness, but extensive scarring of a quarter may render it unproductive in subsequent lactation (Garcia, 2004).

Radostits *et al.,* 2000 explained that, the main source of infection is the udder of infected cows, although when hygiene is poor, contamination of the environment may provide an additional source. The teat and skin of cattle, milkers hand, floors, utensils and cloths are often heavily contaminated. Sores on the teat are the commonest sites outside the udder for the persistence of the organism.

Antimicrobial therapy is a primary tool for controlling streptococcal and staphylococcal mastitis. The efficacy of bovine mastitis treatment depends on the cause, clinical manifestation; antibiotic susceptibility of aetiological agent and the efficacy of immunological system. Mastitis therapy is commonly unsuccessful due to pathological changes that occurs in the glandular tissue as a result of the inflammatory reaction, mastitogenic bacteria related factors, pharmacokinetics properties of antimicrobials drugs, poor animal husbandry and inadequate veterinary services (Maran, 2005).

The emergence of antimicrobial resistance stains of streptococcus and staphylococcus organisms to most commercially available antimicrobial agents in the field (DANMAP, 2001). Furthermore, the knowledge about the pharmacology and susceptibility of antimicrobial agents helps the veterinarians in selecting the most appropriate microbial products for treatment of streptococcus and staphylococcus mastitis (Pol and Ruegg 2007). Resistance to commonly used antimicrobials is frequently encountered with *Streptococci species, Staphylococcus aureus* and coagulase negative staphylococcus. Cure rates of *Staphylococcus au*reus infections are poor after antibiotic treatment (Luthje and Schwarz, 2006).

The main reason of low efficacy of antibiotic treatment of staphylococcal mastitis is among others the resistance of bacteria. Moreover, during past decade, bacteria that cause human diseases have developed resistance to many of the antibiotics commonly used for treatment (Zadoks *et al*., 2000; Zadoks *et al*., 2002; Ekin and Gurturk 2006). The purpose of this work was to determine the invitro activity of antimicrobial agents against Streptococci species, *Staphylococcus aureus* and coagulase negative staphylococcus isolated from clinical cases of mastitis in selected dairy herds in Nigeria.

2. Material and Method

A total of 130 Streptococcal (comprising of 66 from Oyo state, 36 from Kwara state and 28 from Kaduna state) and 177 Staphylococcal (comprising of 107 from Oyo state, 41 from Kaduna state and 29 from Kwara state) isolates were recovered from cases of clinical bovine mastitis. The Streptococcal belonged to six *Streptococci species* namely: St*reptococcus uberis, Streptococcus agalactiae, Streptococcus dsygalactiae, Streptococcus bovis, Streptococcus zooepidemicus, Streptococcus equinus* that were characterized biochemically and Serologically grouped by using a commercial latex agglutination kit for the identification of streptococcal groups A, B, C, D, F and G. The Streptococci were tested using the broth method described by the manufacturer (Oxoid). These streptococcal organisms, *Staphylococcus aureus* and coagulase negative staphylococcus were studied for antibiotic susceptibility to ampicillin, tetracycline, sulphadimidine, streptomycin, neomycin and nalidixic acid (0btained from Sigma Aldrich Chemical Ltd, USA).

Determination of drug resistance (minimum inhibition concentration)

The minimum inhibition concentrations (MIC) of 6 antibiotics namely ampicillin, tetracycline, sulphadimidine, streptomycin, neomycin and nalidixic acid for each of the one hundred and thirty streptococcal and one hundred and seventy seven Staphylococci isolates was determine respectively by tubes method as previously described by Rollins *et al.*, (2003).

A known weight of each antibiotic powder named above except Nalidixic acid was respectively dissolved in sterile Tryptose soy broth (TSB) to a final concentration of 20µg/ml. Nalidixic acid was dissolved with 2drops of 0.2M NaOH and made to a final concentration of 20µg/ml. Oxford *Staphylococcus aureus* NCTC 6571 was used as control organism. All other procedures were as described by Rollins *et al.*, (2003). The MIC of the respective antibiotic was taken as the lowest concentration of the antibiotic that inhibits the growth of the Streptococci isolates. The tubes with clear solution were scored positive (+) that is, the organism did not grow or the organism is sensitive to the antibiotics but the tubes with sediments or growth were scored negative (-) that is, the organism was present or show resistant to the antibiotics. The tubes nearest to where there were sediments or growth were taken as the minimum inhibitory concentration of the antibiotic tested.

3. Results

The antibiotic resistance in *Streptococci species* and *Staphyloccoci species* isolated from clinical mastitic cows in the three states were variable as shown

in Table 1a and 1c below. From Oyo State, All the 47 *Streptococcus uberis* isolates were resistant to ampicillin, neomycin, sulphadimidine, tetracycline while streptomycin and nalidixic acid showed (80.85%) and (0%) resistance respectively. The Streptococcus agalactiae (n=19) displayed 100%, 100%, 100%, 100%, 73.68%, and 0% resistance to ampicillin, tetracycline, sulphadimidine, neomycin, streptomycin and nalidixic acid respectively. Other groups of streptococcus were not found in this state. However, all Staphylococcus aureus (n=98) 100% isolates were resistant to ampicillin, tetracycline, neomycin and sulphadimidine while streptomycin and nalidixic acid displayed 96.94% and 55.10% resistance respectively. For coagulase negative staphylococcus (n=9) displayed 100%, 100%, 100%,77.78%, 77.78% and 22.22% resistance to sulphadimidin, neomycin, tetracycline, ampicillin, streptomycin and nalidixic acid respectively. Tables 1a, 1b and 1c.

In Kwara State, For Streptococcus uberis (n=16) displayed 100%, 100%, 100%, 100%, 62.50% and 50% resistance to sulphadimidine, ampicillin, tetracycline, neomycin, streptomycin, nalidixic acid and respectively. The Streptococcus agalactiae (n=8) displayed 100%, 100%, 100%, 100%, 100% and 0% resistance to sulphadimidine, ampicillin, tetracycline, nalidixic acid, neomycin and streptomycin respectively. Meanwhile, *Streptococcus dysgalactiae* (n=8) showed 100%, 100%, 100%, 75%, 62.5% and 0% resistance to sulphadimidine, ampicillin, tetracycline, neomycin, nalidixic acid and streptomycin respectively. For *Streptococcus zooepidemicus* (n=2) showed 100%, 100%, 100%, 100% , 50% and 50% resistance to sulphadimidine, ampicillin, tetracycline, neomycin, nalidixic acid and streptomycin respectively. *Streptococcus bovis* (n=2) showed 100%, 100%, 100%, 100% , 50% and 50% resistance to sulphadimidine, ampicillin, tetracycline, neomycin, nalidixic acid and streptomycin respectively. *Streptococcus equinus* was not found in Kwara state. However, all *Staphylococcus aureus* (100%) isolates were resistant to ampicillin, tetracycline, sulphadimidine while nalidixic acid Neomycin and streptomycin displayed 85.71% 14.29% and 14.29% resistance respectively. For coagulase negative staphylococcus (n=8) displayed 100%, 100%, 100%, 75%, 12.5% and 0% resistance to sulphadimidine, tetracycline, nalidixic acid, ampicillin, streptomycin and neomycin respectively. Tables 1a, 1b and 1c.

In Kaduna State, isolates of *Streptococcus uberis* (n=9) displayed 100%, 100%, 100%, 44.44%, 33.33%, 0% resistance to sulphadimidine, tetracycline, ampicillin, nalidixic acid, neomycin and streptomycin respectively. For *Streptococcus agalactiae* (n=5) displayed 100%, 100%, 100%, 40%, 0% and 0% resistance to sulphadimidine, ampicillin, tetracycline, neomycin, nalidixic acid and streptomycin respectively. *Streptococcus dysgalactiae* (n=8) showed 100%, 100%, 100%,62.5%, 62.5% and 37.50% resistance to sulphadimidine, ampicillin, tetracycline, nalidixic acid, neomycin and streptomycin respectively. For *Streptococcus zooepidemicus* (n=3) had resistance of 100%, 100%, 66.66%, 66.66%, 33.33%, 0% to ampicillin, tetracycline, sulphadimidine, streptomycin, neomycin and nalidixic acid respectively. *Streptococcus bovis* (n=1) showed 100%, 100%, 100%, 0%, 0%, 0%, resistance to ampicillin, tetracycline, sulphadimidine, neomycin, nalidixic acid and streptomycin respectively. *Streptococcus equinus* (n=2) showed 100%, 100%, 100%, 50%, 0% and 0% resistance to ampicillin, tetracycline, sulphadimidine, neomycin, nalidixic acid and streptomycin respectively. However, *Staphylococcus aureus* displayed 100%, 100%, 100%, 100%, 87.88% and 12.12% resistance to tetracycline, sulphadimidine, neomycin, nalidixic acid, ampicillin and streptomycin respectively while coagulase negative staphylococcus showed 100%, 100%, 100%, 100%, 50%, and 0% resistance to tetracycline, sulphadimidine, neomycin, nalidixic acid, ampicillin and streptomycin respectively. Tables 1a, 1b and 1c.

The overall resistance displayed by all the isolates against the tested antibiotic were highest for tetracycline (100%) followed by sulphadimidine (99.35%), ampicillin (96.09%), Neomycin (85.66%), streptomycin (56.35%) and Nalidixic acid (50.49%) respectively as shown in table II. The resistance patterns exhibited by all the strains revealed 10 distinct resistance groups (Table III). These resistance patterns were demonstrated as triple resistance 33(10.75%), quadruple resistance 42(13.68%), quintuple resistance 157(51.14%) and sextuple resistance 75(24.43%) (Table IV).

Table Ia: Distribution of Antibiotic Resistant Streptococcal species Bacterial Isolates from Mastitic Cows in Nigeria

Antibiotic	% resistant *Streptoccocus agalactiae*			% resistant *Streptoccocus dysgalactiae*			% resistant *Streptoccocus uberis*		
	Oyo (n=19)	Kwara (n=8)	Kaduna (n=5)	Oyo (n=0)	Kwara (n=8)	Kaduna (n=8)	Oyo (n=47)	Kwara (n=16)	Kaduna (n=9)
Ampicillin	100	100	100	0	100	100	100	100	100
Tetracycline	100	100	100	0	100	100	100	100	100
Streptomycin	73.68	0	0	0	0	37.5	80.85	62.5	0
Sulphadimidin	100	100	100	0	100	100	100	100	100
Neomycin	100	100	40	0	75	62.5	100	100	33.33
Nalidixic acid	0	100	0	0	62.5	62.5	0	50	44.44

Table Ib: Distribution of Antibiotic Resistant Streptococcal species Bacterial Isolates from Mastitic Cows in Nigeria

0.	% resistant *Streptoccocus zooepidemicus*			% resistant *Streptoccocus bovis*			% resistant *Streptoccocus eqinus*		
	Oyo (n=0)	Kwara (n=2)	Kaduna (n=3)	Oyo (n=0)	Kwara (n=2)	Kaduna (n=1)	Oyo (n=0)	Kwara (n=0)	Kaduna (n=2)
Ampicillin	0	100	100	0	100	100	0	0	100
Tetracycline	0	100	100	0	100	100	0	0	100
Streptomycin	0	50	66.66	0	50	0	0	0	0
Sulphadimidine	0	100	66.66	0	100	100	0	0	100
Neomycin	0	100	33.33	0	100	0	0	0	50
Nalidixic acid	0	50	0	0	50	0	0	0	0

Table Ic: Distribution of Antibiotic Resistant Staphyloccocal species Bacterial Isolates from Mastitic Cows in Nigeria

Antibiotic	% resistant *Staphyloccocus aureus*			% resistant *Staphyloccocus coagulase negative*		
	Oyo (n=98)	Kwara (n=21)	Kaduna (n=33)	Oyo (n=9)	Kwara (n=8)	Kaduna (n=8)
Ampicillin	100	100	87.88	77.78	75	50
Tetracycline	100	100	100	100	100	100
Streptomycin	96.94	14.29	12.12	77.78	12.5	0
Sulphadimidine	100	100	100	100	100	100
Neomycin	100	14.29	100	100	0	100
Nalidixic acid	55.10	85.71	100	22.22	100	100

Table II: Total number of *Streptoccocal spp* and *Staphyloccocal spp* isolated showing resistance to Antibiotics.

Antibiotic agents	Number of *Streptoccoci spp* (%) resistance	Number of *Staphyloccoci aureus* (%)resistance	Number of coagulase negative Staph (%) resistance	Number of bacterial isolates resistance (%)
Ampicillin	130 (100%)	148 (97.37%)	17 (68%)	295(96.09%)
Tetracycline	130 (100%)	152 (100%)	25 (100%)	307(100%)
Sulphadimidine	128 (98.46%)	152 (100%)	25 (100%)	305(99.35%)
Neomycin	112 (86.15 %)	134 (88.16%)	17 (68%)	263(85.66%)
Streptomycine	69 (53.08%)	102 (67.11%)	8 (32%)	173(56.35%)
Nalidixic acid	32 (24.62%)	105 (69.08%)	18 (72%)	155(50.49%)

Table: 1II The Resistance Pattern for *Streptococcal and Staphyloccocal spp* isolated from cases of clinical mastitis in Nigeria.

Resistant Pattern	*Strep. Agalactiae*	*Strep. Uberis*	*Strep. Dysgalactiae*	*Strep. Bovis*	*Strep. zooepidemicus*	*Strep. Equines*	*Staphy aureus*	*Staphy Coagulase Negative*
PNTeSu	3	4	3	1	1	1	15	5
PNTeSN	0	0	0	0	2	0	0	0
PNTeSuN	7	10	1	0	0	1	0	2
TeSuNNa	0	0	0	0	0	0	4	8
PNTeSuNa	0	2	2	0	1	0	0	2
PNTeSuSN	14	38	0	0	0	0	31	0
PNTeSuNNa	8	8	7	1	0	0	31	0
PNTeSSuNa	0	2	0	0	0	0	3	1
PNTeSSuN	0	8	2	1	1	0	1	0
PNTeSSuNNa	0	0	1	0	0	0	67	7

PN= Ampicillin; Te= Tetracycline; Su=Sulphadimidine; Na=Nalidixic acid;
N= Neomycin

Table: IV Summary of the resistance pattern for *Streptococci spp and Staphylococci spp* isolated from cases of clinical mastitis in Nigeria

Resistance pattern.	*Strept uberis.*	*Strept agalactiae*	*Strept dysgalactiae*	*Strept zooepidemicus*	*Strept. bovis*	*Strept. equinus*	*Staphy aureus*	*Coagulase negative staphy*
Mono resistance	0	0	0	0	0	0	0	0
Double resistace	0	0	0	0	0	0	0	0
Triple resistance	4	3	3	1	1	1	15	5

Quadruple resistance	12	7	3	3	0	1	4	12
Quintuple resistance	56	22	9	1	2	0	66	1
Sextuple resistance	0	0	1	0	0	0	67	7

4. **Discussion**

Antibiotic resistance is an increasing concern worldwide, and there is an agreement that improved surveillance is needed (Anon, 1998, Livermore and Chen, 1999). *In vitro* susceptibility testing is one of the most important functions of diagnostic laboratory. One of the presumed advantages of in vitro dynamic models is the ability to compare pharmacokinetically different antimicrobials (Firsov *et al*., 1999). Information on the amount of antimicrobial agents used in dairy cows and on treatment procedures applied by practitioners and farmers are limited (DANMAP, 2003). Although the overall use of antimicrobial agents is lower in dairy cows than it is in pork or poultry industry (DANMAP, 2003).

From this study, it was observed that all the Streptococci isolates show high rate of resistance to Ampicillin, Tretracycline, Neomycin and Sulphadimidine while greater number were susceptible to Streptomycin and Nalidixic acid. Acikgoz *et al*., (2004) reported high rate of tetracycline resistance (100%) in Turkey Group B haemolytic streptococci especially *Streptococcus agalactiae*. This observation is similar to previous reports (>80% in Canada, 89.1% in France, 87% in Spain and 99.2% in Taiwan) Similarly, 87.5% of 244 isolates of Group B streptococci during 1970 – 1975 in Houston, were resistant (Watts *et al.,* 1995) which is also similar to the findings in this study. The high resistance of streptococci to ampicillin in this study calls for attention. Similar Ojo and Falade, (1974) in dairy herds in Nigeria recorded high resistance percentage of streptococci to streptomycin which was contrary to this present study. Streptomycin resistance of bacteria may be due to drug inactivating enzymes which are usually R-plasmid mediated (Anon, 1998; Watts and Salmon, 1997), or due to genetic mutation causing a change in a particular protein of the 30, ribosome submit (Watts and Salmon, 1997). Occasionally, mammary glands may be infected by *Streptococcus species* of human origin such as *Streptoccus pyogene*. The consumption of such infected milk by humans can cause an outbreak of sore throats (Ojo, 1993). In this study the high resistance of most of the *Staphylococcus aureus* to tetracycline and to ampicilin, agrees with the findings of Watts *et al*., (1995) and Watts and Salmon (1997) on the sensitivity of *Staphylococcus aureus* to antibiotics. The resistance of *Staphylococcus aureus* to tetracycline may have been due to impaired uptake of the antibiotic into the cell or due to the development of mutant strains. Resistance due to diminished uptake of antibiotic by the bacteria cell have been reported to be plasmid mediated (Anon, 1986).

Also, the high resistance of the *Staphylococcus aureus* to ampicilin may have been due to production of β-Lactamase by the organism. It has been reported that *Staphylococcus aureus* are the principal Gram-positive bacteria in which β-Lactamase resistance can develop very quickly Watts and Salmon (1997). The resistance of *Staphylococcus aureus* to ampicillin and the moderate sensitivity of coagulase-negative Staphylococcus to ampicilin were similar to the finding of Pyorala and Pyorala (1998). The most effective control of mastitis is to prevent the contamination of the udder and teat end by pathogenic microorganisms so as to avoid the penetration of the teat canal. This can be achieved by proper hygienic conditions of the milking parlour and thorough disinfection of the udder with water, disinfectant and cloth towel to scrub and dry the teat before milking. The milkers hands should be cleaned, the milking machine should be in good conditions and milking should be done properly .The milking parlour should be free of ticks or other organisms that could damage the udder.

Antimicrobial resistance determined in this study was in line with other reports. Interestingly, a higher proportion of ampicillin, tetracycline and sulphadimidine resistance was discovered in most of the isolates. This finding indicates the need for further investigation of the epidemiology of resistance against ampicillin in Staph. aureus and coagulase negative staphylococcus isolated from bovine mammary glands.

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