

Comparative Efficacy Evaluation of Six Brands of Amoxicillin against *S. aureus* Isolated from Subclinical Mastitic Milking Dairy Cows in Bishoftu

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Received date: February 24, 2017; Accepted date: March 06, 2017; Published date: March 11, 2017

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Abstract

Bovine mastitis is inflammation of the mammary glands that interferes with the normal flow and quality of milk. *S. aureus* is the most important pathogen among *Staphylococci* species related to subclinical mastitis (SCM) in dairy cows. Antibiotics must be safe, effective and of acceptable quality to be used in both human and veterinary medicines. The study was aimed to isolate *S. aureus* from California mastitis test (CMT) positive dairy cows and to evaluate the efficacy of the six brands of amoxicillin against *S. aureus*. Purposive sampling with a cross-sectional study design was conducted from February 2016 to April 2016 in five dairy farms found in Bishoftu town. A total of 162 dairy cows were examined using California mastitis test (CMT) and out of this 112 (69%) were found positive and from this 30 (26.78%) isolates of *S. aureus* were recorded. The highest and the lowest prevalence of the California mastitis test (CMT) positive was found in Prime (100%) and Tseeday (36.84%) farm respectively. The highest and the lowest prevalence of the *S. aureus* isolate were found in Prime (50%) and college of veterinary medicine and agriculture (10%) farm respectively. *In vitro* drug efficacy against the bacterial isolates was determined by comparing the zone of inhibition obtained from clinical and laboratory standards institute (CLSI) by using disc diffusion method. The comparative efficacy between the brands was evaluated by measuring zone of inhibition and was interpreted as resistant, intermediate and susceptible. *S. aureus* isolate were 100% resistant to the six different brands of amoxicillin. Generally it is concluded that *S. aureus* is among the major causative agent of subclinical mastitis in five dairy farms of the study area. The isolates were also resistance to amoxicillin brands indicating the need of other alternative and effective antibiotics.

Keywords:

Amoxicillin; Bishoftu; Dairy cow; disc diffusion; efficacy; *S. aureus*; Subclinical mastitis

Introduction

Ethiopia is the richest country with 49.3 million cattle population in Africa and around 42% of the total cattle herds are milking [1]. This huge population could not meet the milk demand because of a number of factors that reduces milk productivity [2]. Bovine mastitis is an infectious inflammation of the mammary glands that interferes with the normal flow and quality of milk caused by *Staphylococci* species leading to severe economic losses [3-5].

The use of antibiotics in animals and human is for the purpose of disease prevention and treatment [6]. Antimicrobial drugs are used to control, prevent, and treat infection and to enhance animal growth and feed efficiency. Approximately 80% of all food-producing animals receive medication for part or most of their lives [7]. Different antibiotics group or brands have different efficacy in different microorganism. This might happen due to many factors of which quality of active ingredient is mentioned. The low quality of active ingredient produced by the manufacturer result low effective drug against microorganisms. In addition to this problem with packaging, transportation, storage conditions and distribution system are some of the factors deserve to be mentioned [8]. The main reasons for poor quality of drug in developing country is the widespread counterfeiting of medicines, decomposition of the active ingredient in drugs, high temperature and humidity of storage, and poor quality assurance

during the manufacture of medicinal products. Developing and developed countries are suffering with the direct and indirect effect of poor quality drugs at high degree [9].

Poor response and increased resistance of antibiotics to several microorganisms mainly to *S. aureus* isolates has been reported [10,11]. The determination of antimicrobial efficacy is required not only for therapy but also for monitoring the spread of resistant strains throughout the populations [12]. Resistance has been observed to most of the antimicrobial agents currently approved for use in human and veterinary clinical medicine. This situation forced clinicians to depend on data from *in-vitro* antimicrobial efficacy testing [13].

Evaluation of some of the marketed products could give an insight as to the quality of products sold and consumed and could lay basis for future corrective measures. To the best of our knowledge there was no study conducted so far aiming at evaluating the level of efficacy and quality of some brands of amoxicillin commonly found in Ethiopia drug markets.

Therefore, the objective of this study was:

- Isolation of *S. aureus* from California mastitis test (CMT) positive dairy cows found in Bishoftu.
- To know the efficacy of the different brands of amoxicillin against *S. aureus*.

Materials and Methods

Study area

The study was conducted in Bishoftu. Bishoftu town is located in East Shoa zone of Oromia regional state, and located at 9°N latitude and 40°E longitudes at an altitude of 1850 meter above sea level in central high lands of Ethiopia. It has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26°C and 14°C respectively, with mean relative humidity of 61.3% [14]. Farmers in the vicinity of Bishoftu use a mixed crop and livestock farming system.

Study animals and antibiotic

All milking dairy cows of five dairy farms namely Genesis, CVMA, Tseday, EMDIDI and Prime found in Bishoftu town were screened for Sub clinical Mastitis (SCM) using CMT and then milk samples were collected from CMT positive cows and further microbiological analysis was performed. Amoxicillin brands namely were also purchased from Veterinary pharmacy (Table 1).

Study methodology

A cross-sectional study with purposive sampling was conducted from February 2016 to April 2016 in five dairy Farms found in Bishoftu town. Microbiological analysis of the sample and efficacy testing of the antibiotic was performed at the Microbiology laboratory of Addis Ababa University College of Veterinary Medicine and Agriculture, Bishoftu.

Media, drugs and reagents

All the chemicals, reagents and antibiotics used were of analytical grade, obtained from veterinary pharmacy and related chemical and media importer companies in Ethiopia. Media used in this study include: Nutrient Agar (oxid), purple agar base (Difeco), Blood Agar (BBL[®], Becton, Dickinson), Mueller-Hinton Agar (BBL[®], Becton Dickinson), Mannitol Salt Agar (Difeco) and Nutrient broth (Oxoid). All media were prepared according to the manufacturer's specification and sterilized at 121°C for 15 min.

Sampling and sample processing

Sample size determination: Since our study was aimed at isolation and efficacy evaluation, we included all (162) milking dairy cows of five dairy farms screened using CMT purposively. From this we took all positive teats for the purpose of isolation and efficacy was also evaluated accordingly.

Sample collection and transportation: The samples were purposively collected from the five farms found in Bishoftu and the udder was first cleaned with soap then dried with clean towels. Teats were cleaned, disinfected with 70% alcohol before the foremilk from each cow's udder was discarded and the milk was collected by the application of CMT paddle which has four shallow cups marked A,B,C and D for easy identification of the individual quarter from which the milk was obtained. Equal amount of milk and CMT reagent was mixed in the CMT paddle and gently shake to see the gel formation. The quarter with CMT positive was the target milk sample for collection. After collection of the milk sample, all samples were clearly labelled with the

appropriate identification as date of collection, quarter and cow identification, using permanent marker on the Universal bottle. All samples were transported with in an icebox containing ice packs and taken immediately to the Laboratory of Microbiology at the College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, without delay for microbiological analysis. Upon arrival, the samples were stored overnight in a refrigerator at 4°C until examined on the next day. The bacteriological media used in different stages were prepared according to the manufacturer's recommendation.

Bacteriological examination: Isolation and identification of *S. aureus* was conducted in the Microbiology Laboratory of the College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu. The bacteriological culture was performed following the standard microbiological technique recommended by Quinn et al. [15], a loop full of milk was streaked on sterile 5% sheep blood agar and the plates were incubated aerobically at 37°C and examined after 24-48 h of incubation for growth. The colonies were provisionally identified based on Colony morphology, and hemolytic pattern. The representative colonies were sub cultured on mannitol salt agar plate and incubated at 37°C for 24 h. Pure colonies were preserved and maintained for characterizing the isolates on nutrient slants. Thereafter, both primary and secondary biochemical tests were done for identification according to Quinn et al. [15].

Efficacy test and evaluation of six different brands of amoxicillin

Six brands of Amoxicillin approved for use in human medicine were used for efficacy evaluation (Table 1).

Brands	Concentration	Manufacturer/company name	Country
Amoxicillin	500 mg	EPHARM	Ethiopia
Amoxid	500 mg	APF	Ethiopia
Amoxapen	500 mg	Remedica	Cyprus
Amoxil	500 mg	Glaxosmithline	United kingdom
AMYN	500 mg	Kopran	India
Amoxi-denk*	500 mg	Allemagna	Germany

Table 1: Brands of amoxicillin tested for the evaluation of their efficacy. EPHARM=Ethiopian pharmaceutical manufacturing, APF=Addis pharmaceutical factory plc, mg=milligram, *clavulanate added.

Preparation of amoxicillin disks

Whatman No.1 filter papers were obtained and disks of about 6 mm were cut out from the filter papers. These were wrapped in foil paper and sterilized in the oven at 160°C for 1 h. The disks were prepared according to the National Committee for Clinical Laboratory Standards (NCCLS) subcommittee standards and guidelines [16] to contain the concentrations 25 µg equivalent to the standard commercial disk of amoxicillin. Different brands of amoxicillin were diluted to obtain the concentrations of the commercial standard disks using sterile distilled water. First 100 mg powder of amoxicillin was weighed and dissolved in (2000 µl) of distilled water to prepare the stock solution. Then the concentration of the antibiotic becomes 100 mg/2000 µl to reach 50 µl of stock solution. This was diluted by 2000 µl

of distilled water to obtain the standard concentration of the disc. The second concentration was found by the calculation of (volume: concentration) relationship of the given drug. In this condition the concentration was 1.25 µg/µl, but the antibiotic disc has the ability to absorb 20 µl of the solution which contains antibiotic concentration of 25 µg equivalent to the standard.

Disc diffusion test

The antibiotic efficacy patterns of the isolates to different brands of amoxicillin sold in the Ethiopia markets were evaluated using the agar-disk diffusion method on Mueller-Hinton agar according to the National Committee for Clinical Laboratory Standards [16] and Manual of Antimicrobial Susceptibility Testing guidelines [17]. Disc diffusion testing was performed for *S. aureus* isolates in accordance with the Clinical and Laboratory Standards Institute [18]. For these six brands of amoxicillin, prepared unit discs were applied. Finally, the diameters of the zone of inhibition around the disks were measured to the nearest mm using ruler, and the isolates were classified as susceptible, intermediate and resistant according to the interpretative standards [18,19].

Here the *S. aureus* isolates were streaked on nutrient agar and after 24 h a nutrient broth was prepared for the disk diffusion test. Colonies of the *S. aureus* isolates were inoculated into 5 ml of the broths and incubated at 37°C. A turbidometer was used to monitor the turbidity of the broth cultures. Immediately the turbidity exceeded the barium sulfate (McFarland) standard, the incubation was stopped. The broth culture was then diluted 1:10 with a freshly prepared Nutrient broth to give amount of approximately 1 × 10⁵ colonies per ml. Within 20 min of growth reaching final turbidity, each of the isolates was uniformly and aseptically inoculated into a different Mueller-Hinton agar plates by spread plate method using sterile cotton wool. A sterile cotton wool was allowed to soak in the broth culture, squeezed by the side of the bottle before streaking over the sensitivity plates. The appropriate prepared antibiotic disk was aseptically placed on the agar using sterile forceps. The plates were then incubated at 37°C for 24 h, and then measured for diameter of the zones of inhibition [16,17].

Statistical Analysis and Results

The Data was analyzed using descriptive statistics to compute the percentage of CMT positive cows and *S. aureus* isolates.

Results

Descriptive analysis of CMT and bacterial isolates

Farm CMT and cultural prevalence of *S. aureus*: A total of 162 dairy cows were examined using CMT and out of this 112(69%) were found positive and from this 30 (26.78%) isolates of *S. aureus* were recorded.

Farm name	Total screen animal	CMT positive	<i>S. aureus</i> isolate
Genesis	45	27 (60%)	5 (18.5%)
EMDIDI	42	34 (80.95%)	6 (17.64%)
Prime	34	34 (100%)	17 (50%)
Tseday	19	7 (36.84%)	1 (14.24%)
CVMA	22	10 (45.45%)	1 (10%)

Total	162	112 (69%)	30 (26.78%)
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Table 2: CMT positive and *S. aureus* isolates of five farms.

The result indicated that the highest prevalence of CMT positive cows were found in the Prime farm which is almost 100% and the lowest is found in the Tseday farm, which is 36.84%. The highest and the lowest prevalence of the *S. aureus* isolate was found in Prime 17 (50%) and CVMA (10%) farm, respectively (Table 2).

Comparative efficacy evaluation of amoxicillin against *S. aureus*

The efficacy of six brands of amoxicillin commonly sold were evaluated using commercially prepared *S. aureus* test organism (S.COM) (purchased from National veterinary institute) as well as with two isolates of *S. aureus* from the study area which are designated as LH and RH and were representative of the other isolates. Here the standard organism used was not known about its sensibility to Amoxicillin. Depending upon zone of inhibition, Amoxil has very low zone of inhibition in diameter (6 mm) for the commercially prepared *S. aureus* in comparison to the standard amoxicillin disk, which is ≥ 20mm diameter zone of inhibition. Amoxapen has highest zone of inhibition for our isolates designated as LH (left hind quarter), which is 17.66 mm relative to Amoxil. The rest of the brands zone of inhibition lies in between Amoxil and Amoxapen. This result shows that none of the brands have had reached the standard zone of inhibition of amoxicillin against *S. aureus* which is ≥ 20 mm [15,16] (Table 3).

Isolate	A	B	C	D	E	F
S.COM	12 mm	10.33 mm	13 mm	6 mm	13 mm	14 mm
RH	16 mm	15.33 mm	16 mm	15 mm	13.66 mm	14.33 mm
LH	16.33 mm	15.66 mm	17.66 mm	15.33 mm	15.66 mm	15.66 mm

Table 3: Different brands of amoxicillin and their zone of inhibition (n=3). Key: (A=Amoxycillin), (B=Amoxid), (C=Amoxapen), (D=Amoxil), (E=AMYN), (F=Amoxi-Denk), (S.COM=commercially prepared *S. aureus*), (RH=right hind), (LH=left hind).

Discussion

Bacterial, mycoplasmal, and yeast pathogens are the major causative agents of mastitis in dairy cows and the predisposing factors are known to hasten the entry of infectious agents [20]. Among Bacterial infection *Staphylococcus* species are the most common cause of SCM in dairy cows [21,22].

In the present study detailed investigation was carried out for the isolation and identification of *S. aureus* from dairy cows which are found in Bishoftu using CMT screening and cultural isolation. The overall CMT prevalence of SCM (69%) at cow level in this study was in agreement with previous finding of Zerihun [23]; Geresu [24] in Addis Ababa, Mekibib et al. [25] in Holeta, Tolla [26] in South Wello, Abaineh [27] in Fiche et al. [22] in Adama. However, the finding was lower than the previous finding of Nesru [28] in Dire-Dawa. This variability could be attributed due to differences in farm management practice or differences in study methods, agro-climatic condition,

breeds of cows and level of production. High prevalence of mastitis was reported in Farms who were using machine milking than hand milking [29]. This was in agreement with our finding with a high prevalence of CMT positive in prime farm.

The overall prevalence of *S. aureus* isolates from CMT positive cows in this study was 26.78% which was higher than 22.7% of EL-jakee et al. [30] in Egypt and lower than that of Hallén-Sandgren (2000) [31], who reported (37%) in Sweden, Giannecchini et al. [32] (62.8%) in Uruguay, Gundogan et al. [33] 56% in Turkey, Begum et al. [34] 75% in Bangladesh and Zafolon et al. [35] 54.4% in São Paulo. The difference could be due to number of sample involved, hygiene, and the technique of sampling and grade of udder inflammation.

In our study we evaluated six different brands of Amoxicillin (Amoxycillin, Amoxid, Amoxapen, Amoxil, AMYN, and Amoxi Denk) against the commercially available and our *S. aureus* isolates. In our study the isolated *S. aureus* and commercially available were 100% resistant to the different brands of Amoxicillin which was in agreement with the previous finding of Jahan et al. [36], who reported 100% resistance in Bangladesh. These findings were slightly correlated to De Oliveira et al. [37] and Guerin et al. [38], where they analyzed 119 isolates of *S. aureus* collected between 1998 and 2000 in France from cows with mastitis. In an another study in Bangladesh conducted by Begum et al. [34] revealed that *S. aureus* was 37.14% resistant to Amoxicillin; however, in our study we noticed that the antibiotics were 100% resistant to *S. aureus*, indicating increasing resistance of the organism against Amoxicillin. Similar type of resistance patterns were also reported by Islam et al. [39,40]. This variation could be due to decomposition of the active ingredient in drugs, high temperature and humidity of storage, and poor quality assurance during the manufacture of medicinal products, packing and transportation of the drugs [9,41,42].

Conclusion and Recommendation

The present study was conducted to evaluate and compare the efficacy of six different brands of amoxicillin, sold in Ethiopian drug markets; against *S. aureus* bacteria isolated from SCM dairy cows. Amoxicillin is the beta lactam group. High prevalence of SCM in dairy herd is the biggest health and production problem on dairy farms. Generally it is concluded that *S. aureus* is among the major causative agent of SCM in five dairy farms of the study area. The isolates were also resistant to six amoxicillin brands sold in Ethiopia.

Based on the above conclusion the following points are forwarded:

- Different epidemiological factors that are important in SCM occurrence should be studied.
- Awareness should be made for dairy farm owners, milkers and veterinarians on SCM.
- The quality and efficacy of distribution and importation of drugs should be evaluated and controlled in detail by the authorized body periodically.

Further country wide investigation should be performed concerning the resistance pattern of *S. aureus* against amoxicillin.

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