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Journal for Young Scientists
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Antibiotic susceptibility and determination of Minimum Inhibitory Concentration (MIC) of potent antibiotics used against *Staphylococcus* spp. isolated from raw milk

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Received : December 2010
Accepted : November 2011
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Abstract : *The uncontrolled use of antibiotics has led to the development of multiple antibiotic resistance, there by rendering the treatment ineffective. In the present study, raw milk samples were collected from different areas of Patna. Out of the 12 isolates obtained, nine were identified as Staphylococcus species. The isolates were examined for their susceptibilities by Bauer Kirby Disc Diffusion test against ten antibiotics. Results showed that incidence of resistance to the antibiotics was quite high, as the maximum susceptibility obtained was only about 13.19%, Rifampicin and Tetracycline being the most ineffective in vitro. Amoxicillin and Cloxacillin were the most effective in phase I exhibiting 12.13% and 11.24% efficacy, respectively, while Ampicillin + Cloxacillin was the most effective combination exhibiting 14.31% efficacy in phase II. The MIC values of two antibiotics in pure form and three in combinations were determined by agar dilution and broth dilution methods. The MIC values ranged between 0.5 – 1.0 µg/L showing comparable results throughout the dilution range. However, slightly higher values were obtained for Amoxicillin + Erythromycin and Amoxicillin + Clavulanate i.e. $\geq$ 1.0 µg/L.*

Keywords: *Antibiotic efficacy, Staphylococcus, MIC values.*

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Introduction :

Milk is an excellent culture medium for different kinds of microorganisms, being high in moisture, nearly neutral in pH, and rich in nutrients like lactose, citrate, butter fat and proteins. *Staphylococcus species* are known to be the common contaminants of milk which cause diseases like Mastitis or the inflammation of udder in cattle. Antibiotics, which are the antimicrobial

chemotherapeutic agents of microbial origin, are commonly used for the treatment of *Staphylococcus* infections including mastitis (Frazier and Westhoff, 2008).

The composition of milk, its natural microbiological contamination and the degree of handling can explain the fact that dairy products are frequently implicated in food-borne diseases. Among pathogenic bacteria, *S.aureus* is one of the most abundant microorganisms isolated from raw milk, and also the microorganism most commonly isolated from bovine mastitis (Devriese *et al*. 1997).

The presence of *S.aureus* in asymptomatic food handlers is well documented and has been attributed to contamination of dairy products. Irregularities in storage time and temperature, and failures in the hygienic procedures during the production of dairy products, are factors that may lead to high contamination with *S.aureus*. Therefore, even industrialized dairy products can be sources of food intoxication, since the staphylococcal enterotoxin is not inactivated by pasteurization (Peterson and Shanholtzer, 1992).

Antibiotics are used to treat diseases of cattle and as preservatives for milk. The uncontrolled use of antibiotics has led to the development of multiple antibiotic resistance, there by rendering the treatment ineffective. Thus, there is an urgent need to study and uncover the recent trends in resistance (Devriese *et al*. 1997).

Consumption of milk having significant amount of antibiotic without proper antibiotic flush, results in accumulation of these antibiotics in humans. Moreover, several resistant Staphylococcus may also enter the body and can cause infections like impetigo, cellulitis, food poisoning, scalded skin syndrome, toxic shock etc. MIC can reveal the lowest concentrations, which leads to a reduction in the antibiotic load passing on from the milk to the body.

Minimum inhibitory concentration is the lowest concentration that is able to inhibit growth of the bacteria. MIC's are used by diagnostic laboratories, mainly to confirm resistance and as a research tool to determine the *in-vitro* activity of antibiotics. It is also used to decide the appropriate antibiotic concentrations which can be administered at safe clinical or subclinical levels (Andrews, 2006).

Materials and Methods :

The study was conducted in the Industrial Microbiology laboratory, Patna Women's College and Indian Institute of Bioinformatics and Biotechnology, Patna.

Sampling

Ten raw milk samples were collected aseptically in glass vials from five different areas of Patna, namely, Boring Road, Patliputra, Gardanibagh, Patel Nagar and Kankarbagh. The serial decimal dilutions of these milk samples were then prepared in 0.1% peptone, which was supplemented with 0.05% (w/v) Tween-80 and 0.1% (w/v) $MgCl_2.6H_2O$. These dilutions were prepared in duplicates and then transferred to Mannitol salt agar (Dubey and Maheshwari, 2006). All plates were incubated at 37ºC for 48 hours.

Biochemical Characterization and Identification

Typical colonies grown on MSA plates were identified as *Staphylococcus species* using the following tests: Gram staining, IMViC tests, Carbohydrate (dextrose, sucrose and lactose) fermentation, Gelatinase production, Amylase production and Catalase production. The isolates were also tested for production of Coagulase by the Slide Coagulase test and furthur grouped as α, β and γ hemolytic by performing Hemolysis on Blood agar medium (Williams 2000).

Antibiotic susceptibility test (AST) by Bauer Kirby Disc Diffusion Technique

Mueller Hinton Agar is recommended for antimicrobial disc diffusion susceptibility testing of common, rapidly growing bacteria by the Bauer-Kirby method (Barry *et al.* 1992) as standardized by the National Committee for Clinical Laboratory Standards. The tested antibiotics were: Amoxicillin, Erythromycin, Rifampicin, Tetracycline and Cloxacillin. Further, following combinations of antibiotics were also tested: Amoxicillin + Clavulanate, Amoxicillin + Erythromycin, Rifampicin + Erythromycin, Tetracyclin + Cloxacillin and Cloxacillin + Ampicillin (Andrews 2006). Stock solutions of the antibiotics were prepared in the recommended solvents and three dilutions i.e. 50, 100 and 150 µg/ml were prepared from the stocks.

The disc diffusion test was done for each isolate on Mueller-Hinton agar. For this, 25 ml of medium was poured into sterile Petri dishes to a depth of 4 mm on a level surface to make the depth of the medium uniform and left at 37ºC temperature overnight to check sterility (Barry *et al.* 1992). For inoculum preparation 5 ml Tryptic Soy broth was dispensed in 15 ml culture tubes and sterilized by autoclaving at 121°C for 15 minutes.

The tubes were cooled and kept in an incubator for 24 hours at 35°C to check sterility. Then, each isolate was inoculated in the sterilized tubes containing the medium, and placed in an incubator overnight at 35°C. An antibiotic free control was also prepared.

Inoculation of plates

100µL of each isolate suspension was pipetted out through a micropipette and placed on the plates. Then, the inoculum was spread evenly over the surface of the medium using a glass spreader.

Preparation and loading of antibiotic discs

Discs of equal size and uniform shape were punched out of a Whatman filter paper No. 1 and sterilized. The discs were applied by means of a sterile forceps, strictly under aseptic conditions. The discs were deposited onto the plates so that the centers were at least 24 mm apart. Placing discs adjacent to one another was avoided. After discs have been placed on the agar, they were tapped gently with the sterile forceps to make complete contact with the medium surface. A control plate without any antibiotic was also prepared for each isolate. All steps were performed in duplicates (Isenberg, 1988). Within 15 minutes after the discs were applied; the plates were inverted and incubated at 35°C. Plates were examined after 24 hours of incubation.

Inhibition Zone measurement

The measuring scale was held on the back of the inverted plate over a black, non-reflecting background, and the more obvious margin was measured to determine the zone diameter. Growth within the apparent zone of inhibition was indicative of resistance.

Determination of Minimum Inhibitory Concentration (MIC) of potent antibiotics

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation, and minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial that will prevent the growth of an organism after sub-culture on to antibiotic free media (Andrews, 2006). MICs are used by diagnostic laboratories, mainly to confirm resistance, but most often as a research tool to determine the *in-vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints (James, 1990).

Based on the results obtained from the Bauer Kirby antibiotic susceptibility test, the following antibiotics were found to be the most effective and hence selected for determining the MIC values: Amoxicillin, Erythromycin, Amoxicillin + Erythromycin, Amoxicillin + Clavulanate and Ampicillin + Cloxacillin.

The following methods were used to determine the MIC values:

Method I - Agar Dilution Method

Stock preparation:

The standard stock solutions were prepared by choosing a suitable range against *Staphylococcus* spp. To prepare a stock of concentration of 50 mg/ml, 500 mg powder from the capsule was directly weighed and dissolved in 10 ml of solvent. For preparing stock solution of antibiotics in combination 250 mg powder of each antibiotic was weighed and mixed in 10 ml of the solvent. Further, a stock of concentration 10,000 mg/L was obtained by adding 2 ml of 50 mg/ml solution to 8 ml of distilled water. Further stock solutions, from the initial 10,000 mg/L solution, were prepared as follows **:**

1000 μL of 10,000 mg/L solution + 9 mL diluent = 1000 mg/L
100 μL of 10,000 mg/L solution + 9.9 mL diluent = 100 mg/L

Preparation of Antibiotic dilution range:

Dilutions were prepared from the stock according to the target range. The target range of MIC was between 0.25 – 128 mg/L (Andrews, 2006). Thus, this dilution range was selected and prepared as follows: 11 sterilized glass vials were labeled as follows: 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 mg/L.

From the 10,000 mg/L stock, the following amounts were dispensed with a micropipette:

256 μl into the vial labeled 128

128 μl into the vial labeled 64

64 μl into the vial labeled 32

32 μl into the vial labeled 16

From the 1000 mg/L stock, the following amounts were dispensed into the vials:

160 μl into the vial labeled 8

80 μl into the vial labeled 4

40 μl into the vial labeled 2

From the stock 100 mg/L the following amounts were dispensed into the vials:

200 μl into the vial labeled 1

100 μl into the vial labeled 0.5

50 μl into the vial labeled 0.25

No antibiotic was added to the vial labeled 0 mg/L (antibiotic free growth control).

Preparation of inoculum:

5 ml tryptic soy broth was dispensed in 15 ml culture tubes and sterilized by autoclaving at 121°C for 15 minutes (Aneja 2004). The tubes were cooled and kept in an incubator for 24 hours at 35°C to check sterility. Then, a loopful of each isolate was inoculated in the sterilized tubes containing the medium, and placed in an incubator overnight at 35°C.

Inoculation and incubation:

100μl of each isolate suspension was pipetted out through a micropipette and added onto the plates. Then, the inoculum was spread evenly over the surface of the medium using a glass spreader. All plates were incubated under appropriate incubation conditions i.e. at 37ºC for 16 – 18 hours. Thereafter, the plates were observed for growth.

Method II – Broth Dilution Method

(A) Macrodilution

Preparation of antibiotic stocks and dilution:

Antibiotic ranges were prepared which were one step higher than the final dilution range required,

i.e. for a final dilution range of 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 mg/L, a range of 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 mg/L were prepared (to compensate for the addition of an equal volume of inoculum). Sufficient 75 x 12 mm sterile capped tubes were arranged in two rows for each antibiotic, to cover the range of antibiotic dilutions chosen in duplicates. The tubes were labeled with each dilution and 5 ml of Tryptic Soy broth was poured in each tube. 0.25 ml volume of each antibiotic dilution was added to the broth in tubes (Aneja 2004).

Preparation of inoculum:

Normal saline was prepared by dissolving 0.875 g NaCl in 100 ml distilled water and autoclaved. 5 ml of saline was poured into 10 sterile glass vials. A loopful of each isolate was taken and added to the saline in each vial and vortexed to prepare a homogenous suspension.

Inoculation of tubes:

1 ml aliquot of test organism was added to each set of tubes and the contents of the tubes were mixed thoroughly. Two antibiotic free control tubes were also prepared as given in Table 1.

Table 1: Indication and purpose of the control tubes

Control type	Inoculation with isolate	Purpose
Control I	+	Controls the adequacy of the broth to support the growth of the organism.
Control II	-	Check of sterility.

All tubes (including control) were incubated at 35 - 37°C for 18 – 20 hr in air. Spectrophotometric reading of each sample was performed with a spectrophotometer (Thermo) set at 492nm.

(B) Microdilution

Stock solutions, dilutions and broth suspensions were prepared as above. Tryptic Soy agar was prepared (Dubey and Maheswari, 2006). It was poured into 150 mm petriplates and allowed to solidify. After the agar has solidified wells were bore into the plates using a well borer.12 wells were prepared in each row. A total of 96 wells were prepared, 48 on each plate (for each antibiotic in duplicate).75 µl of each antibiotic dilution was added to two rows of wells, except for two wells in each row.75 µl of test organism was dispensed into one row and 75 µl of control into the second row of wells. This included both inoculated and uninoculated wells of antibiotic-free broth (the first controls the adequacy of the broth to support the growth of the organism, the second is for check of sterility).The plates were incubated at 37°C for 18 hours.

A 96 well sterile microtitre tray was labeled from 1 to 12 which represent the appropriate antibiotic dilutions and two controls. The contents of each well from the plates were pipetted out and placed into the wells of the tray. Mixing of the contents was performed in the wells by gently flushing the inoculum in and out of the pipetted tip four to five times, avoiding splashing or creation of bubbles. The tray was covered with a sealing tape and gently agitated. The endpoint reading was performed using the following methods:

(i) Reference MIC endpoint reading (V)

The broth microdilution wells that had not been agitated were read visually (V) with the aid of a reading mirror; the growth in each well was compared with that in the growth control (drug-free) well. A numerical score, which ranged from 0 to 4, was assigned to each well according to the scale recommended by National Committee for Clinical Laboratory Standards (NCCLS) as shown in Table 2.

Table 2: Numerical scores as per NCCLS guidelines

Numerical score	Indication
0	optically clear
1	slightly hazy
2	prominent decrease in turbidity
3	slight reduction in turbidity
4	no reduction in turbidity

(ii) Visual MIC following agitation (VS)

In order to assess alternative methods for the determination of MIC endpoints, the MIC for each drug-organism pair was read visually following agitation of the microdilution trays. Agitation was accomplished by first sealing the tops of the trays with clear tape and then shaking gently until a homogeneous suspension was obtained in each well. The MIC endpoints for the agitated trays (VS) were defined exactly as described above for the reference MICs for the V trays.

Results and Discussion :

A total of 12 isolates were obtained from 10 raw milk samples. Out of the 12 isolates obtained, nine were identified as *Staphylococcus* species. Out of nine, two isolates were coagulase positive and 7 coagulase negative. Also, five were hemolytic and four non hemolytic. The isolates were examined for their susceptibilities by Bauer Kirby Disc Diffusion test. The susceptibility patterns exhibited by the isolates and the efficacy of the antibiotics are shown in Fig 1 and 2.

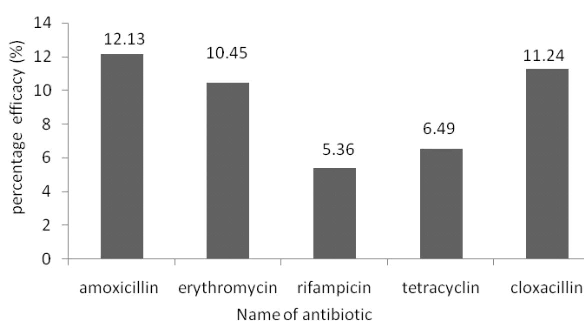

Fig 1: Percentage efficacy of antibiotics in AST phase I

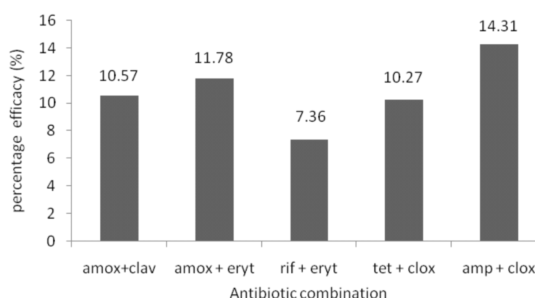

Fig 2: Percentage efficacy of antibiotics in AST phase II

Fig 1 and 2 show that incidence of resistance to the antibiotics was quite high as the maximum susceptibility obtained was only about 14.31%, Rifampicin and tetracycline being the most ineffective *in-vitro*. Amoxicillin and Cloxacillin were the most effective in phase I exhibiting 12.13% and 11.24% efficacy respectively, while Ampicillin + Cloxacillin was the most effective combination in phase II exhibiting 14.31% efficacy. Fig 3 shows the inhibition zones obtained after AST.

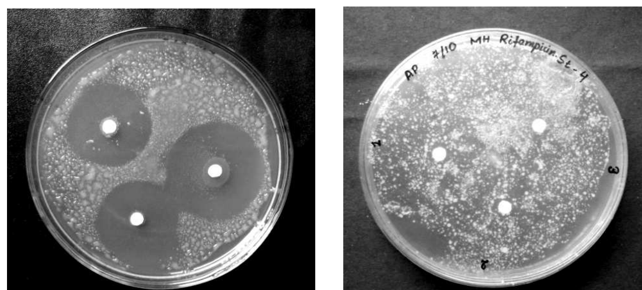

Fig 3: Results of AST: Susceptibility to Amoxicillin (left) and resistance to Rifampicin (right)

Method I - Agar Dilution Method

On an average the MIC values for the antibiotics were recorded between 0.5-1.0 mg/L. The MIC values were found to be slightly greater than those which have been evaluated in the previous years (Andrews, 2006).

Method II – Broth Dilution Method

For Broth Macrodilution the following optical density was recorded :

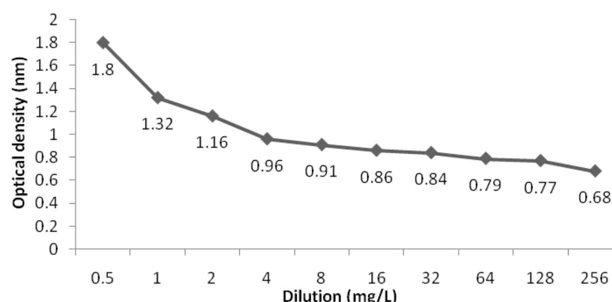

Fig 4: Optical density for Amoxicillin + Cloxacillin by MIC Broth macrodilution method

In the Broth dilution method, the optical density showed a steep decrease even after a single doubling of dilution from 0.5 to 1.0 mg/L. This has been illustrated taking the example of the combination Amoxicillin + Cloxacillin which yielded best results as shown in Fig 4.

For broth microdilution the end point readings were recorded and the numerical scores were assigned according to the NCCLS guidelines (Table 2). Results obtained from both V and VS methods were in accordance with those of NCCLS 2000 as shown in Fig 5.

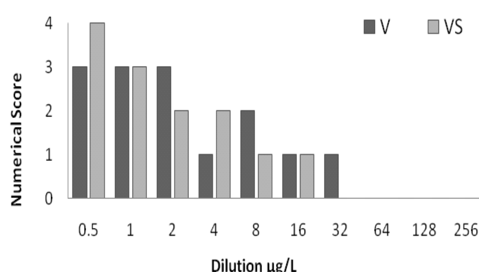

Fig 5: Comparative results of MIC for Amoxicillin + Cloxacillin: V (MIC) and VS (MIC after agetation) endpoint readings

Conclusion :

The present study pointed out that the milk samples were contaminated with *Staphylococcus spp.* probably due to the ineffectiveness of antibiotics used for clinical purposes. It can be said that using antibiotics to declare "all-out war" against bacteria like *Staphylococcus spp.* is a war that we cannot win. Moreover, the indiscriminate use of antibiotics at high concentrations in dairy farms allows significant amounts of antibiotics to enter the human body through the consumed milk, besides Staphylococcal enterotoxins which are resistant even to pasteurization. Antibiotic resistant *Staphylococci* are of great concern both in veterinary and human medicine worldwide. Like other bacterial pathogens many *Staphylococcus spp.* have become resistant to many antimicrobials through the acquisition of mobile drug resistance gene.

Results obtained from the Bauer Kirby Disc Diffusion test or the AST showed that Rifampicin and Tetracycline were most ineffective while Amoxicillin, Cloxacillin and a combination of Ampicillin + Cloxacillin was most effective with 12.13%, 11.24% and 14.31% efficacy, respectively. Results of MIC determination and a statistical and comparative analysis of the data obtained showed that Amoxicillin in pure form and even when present in combination with Erythromycin and Clavulanate may be the most effective antibiotics against *Staphylococcus spp.*

Combinations significantly affected the susceptibility patterns. However, changes for certain combinations could not be traced and hence, further evaluations are needed before clinical trials. The methods used were practical for a clinical laboratory that chooses to perform bactericidal activity testing assuring a high level of reproducibility between duplicate assays.

Acknowledgement :

We are grateful to Dr. Sister Doris D'Souza A.C., Principal, Patna Women's College (PWC) and the Research Committee for providing facilities and financial support. We thank Prof. S. Bedi, Head, Department of Industrial Microbiology, PWC, for taking keen interest in our research work.

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