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EFFICACY AND COMPLICATIONS OF PROLONG LINEZOLID THERAPY IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) INFECTED SUBCUTANEOUS ABSCESS MODEL IN WISTAR RAT

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ABSTRACT: Linezolid has a better choice for the eradication of both community and hospital acquired methicillin-resistant *Staphylococcus aureus* (MRSA) infections, but its use is limited because of its complications. The study elucidated the efficacy and complications of prolonged therapy of linezolid in MRSA infected rats. The rats were rendered neutropenic by an intraperitoneal injection of cyclophosphamide injection given for 4 days and 5th day at a dose of 150 mg/kg and 100 mg/kg, respectively. This neutropenia was maintained for 5 days. The neutropenic rats were injected subcutaneously with 10^6 CFU/ml of MRSA. The rats were divided into 3 groups. Normal control, Infected, Infected animals treated with linezolid 50 mg/kg/twice/day for 14 days. On the 15th day, the blood and liver were collected for biochemical and histopathological examination. The MRSA was confirmed by PCR assay. The minimum inhibitory concentration of linezolid was 0.5-2 µg/ml. The decreased bacterial count (7.22×10^3 CFU / abscess), intestinal alkaline phosphatase (IAP), increased lactic acid, alteration in hematological parameters and liver damage were seen in linezolid treated infected rats when compared to normal animals. Our study concludes the prolonged use of linezolid cause intestinal dysbiosis, myelosuppression, mitochondrial toxicity, and hepatotoxicity.

INTRODUCTION: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a specific gram-positive "Staph" bacteria that is most often causes pneumonia, surgical infections, catheter infections and invasive infections such as soft tissue infections, heart valve infections, bone infections, dental and bloodstream infections [1]. It is often resistant to several types of antibiotic treatments. That makes it harder to treat someone who gets an infection. Stronger, more selective, expensive, and intravenous antibiotics may be needed to treat MRSA infections [2].

Furthermore, currently available antibiotic to treat MRSA infection is limited. Linezolid, tigecycline, clindamycin, vancomycin, and daptomycin showed efficacy, safety, clinical cure and eradication rates in MRSA [3]. The Linezolid (LZD) is oxazolidinone antibiotic superior over vancomycin in the eradication of MRSA [4]. LZD has a unique structure and mechanism of action, which targets protein synthesis at an exceedingly early stage. LZD has 100% bioavailability, when given orally and allows conversion to oral therapy when the patient is

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clinically stable. It reduces the length of hospital stay, cost, intravenous infections and increased patient convenience [5].

Consequently, LZD has no cross-resistance with other commercially available antimicrobial agents. Linezolid-resistant *Staphylococcus aureus* is uncommon, with >99% of isolates being susceptible in surveys [6, 7]. Linezolid is a relatively safe antibiotic when given for short periods. It is well tolerated, with myelosuppression, mitochondrial toxicity being the most serious adverse effect [8]. Prolong LZD therapy is needed for MRSA infections in prosthetic joint and diabetic wound infection. The present study was undertaken to evaluate the efficacy and complications of prolonged LZD therapy in MRSA infected subcutaneous abscess model.

MATERIALS AND METHOD:

Drugs and Chemicals: Linezolid were obtained from Sigma-Aldrich Chemical Pvt. Limited, India. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade. For estimation of biochemical tests, kits were obtained from Ecoline, Manufactured by Merck Specialties, Private limited, Ambernath.

Experimental Animals: The Wistar rats (200-250 g) were maintained in clean, sterile, polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 25 ± 2 °C and humidity of 30-60%. A 12:12 h light and the dark cycle were followed. All the experimental procedures and protocols used in this study were reviewed by the Institutional animal ethics committee (688/2/ CPCSEA) and were by the guidelines of the IAEC. Animal care was given as per the guidelines of Committee for Control and Supervision of Experiments on Animals (CPCSEA).

Microorganism: The clinical isolate of MRSA with positive in both Panton-Valentine leukocidin (Pvl) toxin and methicillin resistance (*mecA*) gene were obtained from the ARM laboratory, Erode, Tamil Nadu, India.

PCR Screening: A duplex-PCR was used to screen the presence of *mecA* and *luk S/F-PV* (which encode the PVL S/F bicomponent proteins) genes [9]. For rapid DNA extraction, 2 - 5 colonies were suspended in 100 µl of molecular-grade water (Qiagen, Germany) and heated in a boiling water bath for 10 min. After centrifugation at 10,000 rpm for 10 min, 1 µl of the supernatant (DNA template) was added to 20 µl of the PCR reaction mixture (Invitrogen, USA). The reaction was carried out using the following conditions: an activation step at 94^{o} C for 5 min, followed by 30 cycles of initial-denaturation at 94 ºC for 30 s, 50 ºC for 30 s, and 72 ºC for 30 s, ending with a final extension step at 72 ºC for 7 min, and followed by a holding step at 4 ºC. The PCR products were analyzed by gel electrophoresis with 2 percent agarose in Tris/Borate/EDTA buffer with ethidium bromide (5 µg/mL) and visualized by UV-transillumination. A 100 bp DNA ladder (Invitrogen, USA) was used as a marker Duplex-PCR yielded the products with sizes of 433 and 310 bp which corresponded to *lukS/F-PV* and *mecA* genes respectively.

Determination of MIC using Broth Microdilution Assays: Microbroth dilution assays were performed according to the Clinical and Laboratory Standards Institute guideline (CLSI) [10]. A single colony was transferred and incubated overnight at 37 °C with Mueller Hinton broth. The diluted culture was measured for the absorbance at OD_{620} to get a concentration of 1×10^6 CFU/ml. A total of 100 of the freshly diluted cultures and serially diluted antibiotics were dispensed into 96-well culture plates. The plates were incubated at 37°C for 16 to 18 h. The last well in the series without any visible growth was read as MIC.

Experimental Design: Rats were rendered neutropenic by intraperitoneal cyclophosphamide injections given for 4 days and 5th day at a dose of 150 mg/kg and 100 mg/kg, respectively. This neutropenia was maintained for 5 days. The neutropenic rats were injected subcutaneously with 0.2 ml of the MRSA suspension containing approximately 10^6 CFU/ml in 5% hog gastric mucin [11]. Animals received treatment of anti-infective compounds starting 2 h post infection. Linezolid was administered orally at a dose of 50 mg/kg twice daily for 14 days [12].

The animals are divided into 4 groups (n=10)

Group I: Control
Group II: Rats were infected with MRSA
Group III: Infected rats treated with linezolid 50 mg/kg twice daily.

On 15th day animals were anesthetized, and blood samples were collected into EDTA added vials for hematological study and into dry non-heparinised tubes for serum biochemical profile, and the serum was separated by centrifuging at 3000 rpm for 15 min. The abscesses were excised, added to 2 ml of heart infusion broth, and homogenized. A standard plate procedure determined the number of viable organisms in the abscess (the number of CFU per abscess) with mannitol salt agar. The liver tissue portions were dissected and then fixed in 10% formalin for histopathological studies. Sections of 5-6 µ thickness were prepared and stained by hematoxylin and eosin staining agents.

Preparation of Intestinal Homogenate: The intestine was removed, flushed with saline and washed again. The mucosa was scraped off with the help of a microscopic glass slide. 10% w/v of the mucosa was mixed with 5 mM EDTA (pH-7.4) and homogenized with Teflon pestle. The homogenate was centrifuged, and the supernatant liquid was used for estimation of intestinal alkaline phosphatase (IAP).

Statistical Analysis: The data are expressed as the mean ± standard deviation (SD). GraphPad Prism software was used to analyze data and construct the graphs. One-way analysis of variance (ANOVA) followed by Dunnett's test. The values of $p<0.05$ were regarded as statistically significant.

RESULTS:

***In-vitro* Antimicrobial Activity:** The clinical isolate of MRSA was confirmed by duplex PCR **Fig. 1**. The clinical and laboratory standards institute clinical breakpoints were considered for interpretation of linezolid MIC (sensitive ≤4 µg/ml and resistant ≥ 8 µg/ml) *S. aureus* ATCC 29213 was used as a control strain for MIC detection. MRSA isolates tested, was sensitive to linezolid ranged from 0.5 µg/ml - 2 µg/ml. linezolid showed well *in-vitro* activity against MRSA isolates and can be considered for the treatment of these infections.

Bacterial Count: The number of viable organisms in the abscess (number of CFU per abscess) showed in **Table 1**. The infected animals showed 4.20×10^6 CFU/abscess **Fig. 2a**. The infected animals treated with linezolid showed a significant reduction (7.22×10^3 CFU/abscess) in the bacterial count when compared to infected animals.

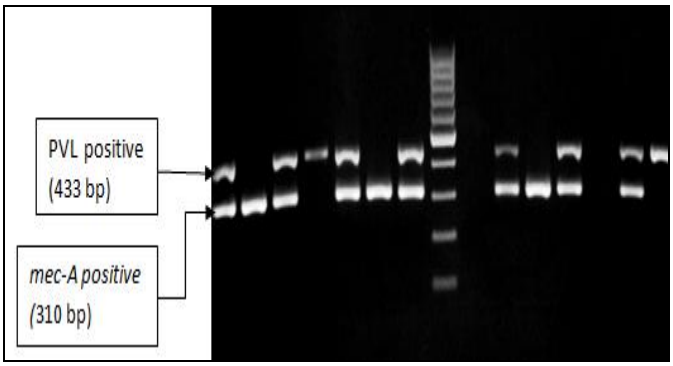

FIG. 1: DUPLEX - PCR FOR PVL AND *MEC A* GENES

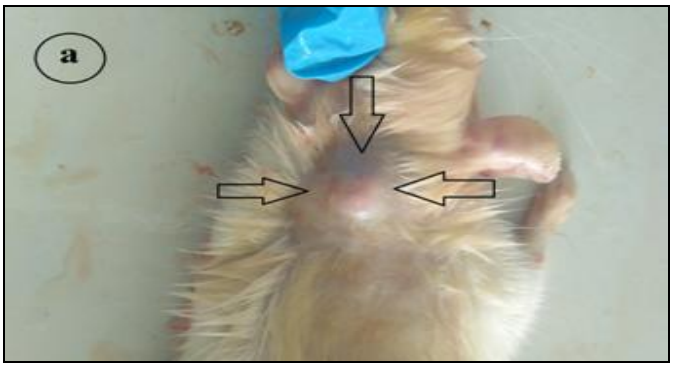

FIG. 2: SHOWED MRSA INDUCED ABSCESS IN WISTAR RATS

TABLE 1: MRSA COUNT OF INFECTED AND LINEZOLID TREATED GROUPS

Group	MRSA CFU/Abscess
Infected (I)	$4.20 \times 10^6 \pm 0.02$
Infected + LZD	$7.22 \times 10^3 \pm 0.01$

Values are mean ± S.D; n=10 in each group

Hematological Parameters: We found a significant reduction ($p<0.01$) in RBC, WBC, PLT, and Hb in linezolid treated infected animals **Table 2** when compared to normal animals. These findings suggest linezolid treated animals exhibited myelosuppression.

TABLE 2: HEMATOLOGICAL PARAMETERS OF NORMAL AND DIFFERENT EXPERIMENTAL GROUPS

Group	RBC ($\times 10^6$/µl)	WBC ($\times 10^3$/µl)	PLT ($\times 10^5$/µl)	Hb (gm/dl)
Normal	6.24 ± 0.2	12.38 ± 0.6	8.28 ± 0.1	12.18 ± 0.3
Infected	6.14 ± 0.3 ^{ns}	13.02 ± 0.1**	7.94 ± 0.3 ^{ns}	12.10 ± 0.2^{ns}
Infected + LZD	4.93 ± 0.1**	10.32 ± 0.2**	4.96 ± 0.2**	10.03 ± 0.1**

Values are mean ± S.D; n=10 in each group; ^{ns} P>0.05 and ** P<0.01, when compared to normal control (one way ANOVA followed by Dunnett's test).

Lipid Profile: The linezolid treated infected rats shown **Fig. 3** no significant (p>0.05) change in the cholesterol and triglycerides in comparison to the normal group.

Serum Hepatic Biomarker: The linezolid treated infected rats shown **Fig. 4** significant (p<0.01) elevation in the AST, ALT, ALP, LDH levels in comparison to the normal group.

It indicates it prolong LZD treatment induce liver damage. The linezolid treated infected animals showed 2.19 mU/mg of IAP represented in **Fig. 5**.

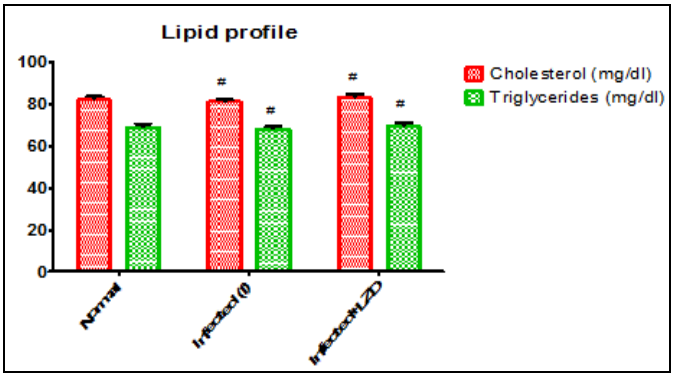

FIG. 3: EFFECT OF LINEZOLID ON LIPID PROFILE IN DIFFERENT EXPERIMENTAL GROUPS
Values are mean ± S. D; n=10 in each group; #P>0.05 when compared to normal control (one way ANOVA followed by Dunnett's test).

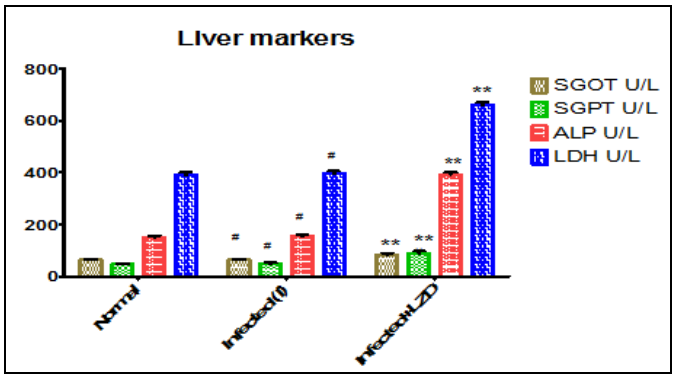

FIG. 4: EFFECT OF LINEZOLID ON LIVER ENZYMES IN DIFFERENT EXPERIMENTAL GROUPS. Values are mean ± S.D; n=10 in each group; #P>0.05 and **P<0.01, when compared to normal control (one way ANOVA followed by Dunnett's test).

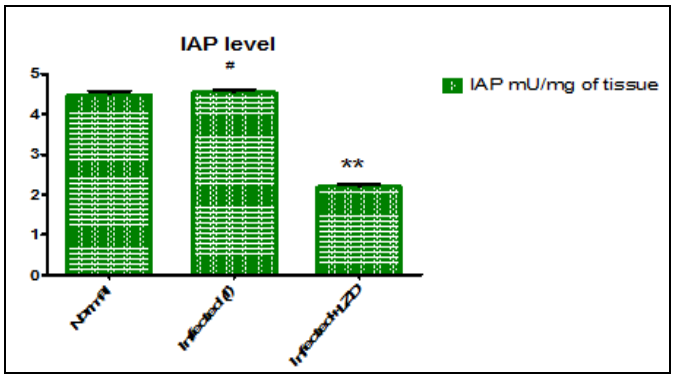

FIG. 5: EFFECT OF LINEZOLID ON IAP LEVEL IN DIFFERENT EXPERIMENTAL GROUPS. Values are mean ± S.D; n=10 in each group; #P>0.05 and ** P<0.01, when compared to normal control (one way ANOVA followed by Dunnett's test).

Serum Biochemical Parameters: LZD treated infected animals showed **Fig. 6** significant (p<0.01) elevation (20.1 mg/dl) of lactic acid and bilirubin indicates liver damage, but no substantial change in urea and creatinine when compared to control. The elevated lactic acid is the marker for mitochondrial toxicity.

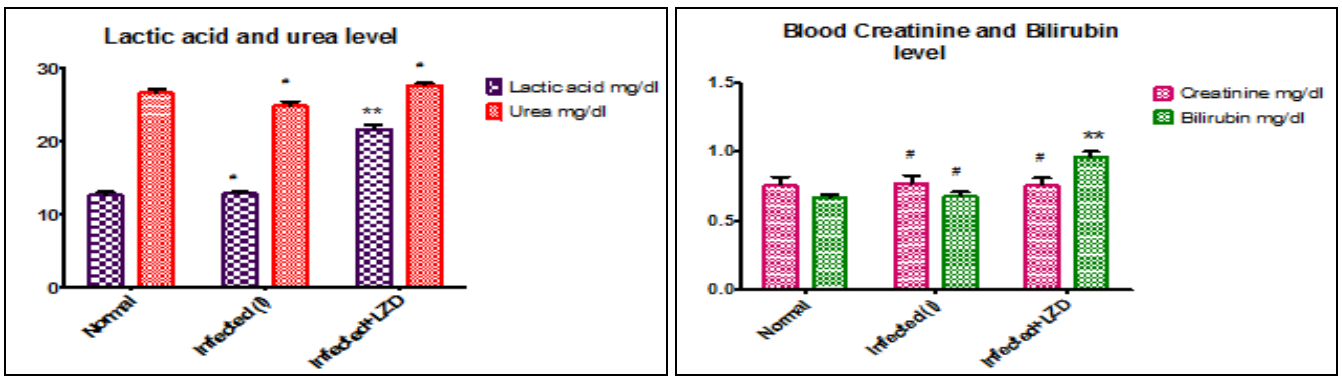

FIG. 6: EFFECT OF LINEZOLID ON LACTIC ACID, UREA, CREATININE, AND BILIRUBIN IN DIFFERENT EXPERIMENTAL GROUPS. Values are mean ± S.D; n=10 in each group; #P>0.05, ** P<0.01, when compared to normal control (one way ANOVA followed by Dunnett's test).

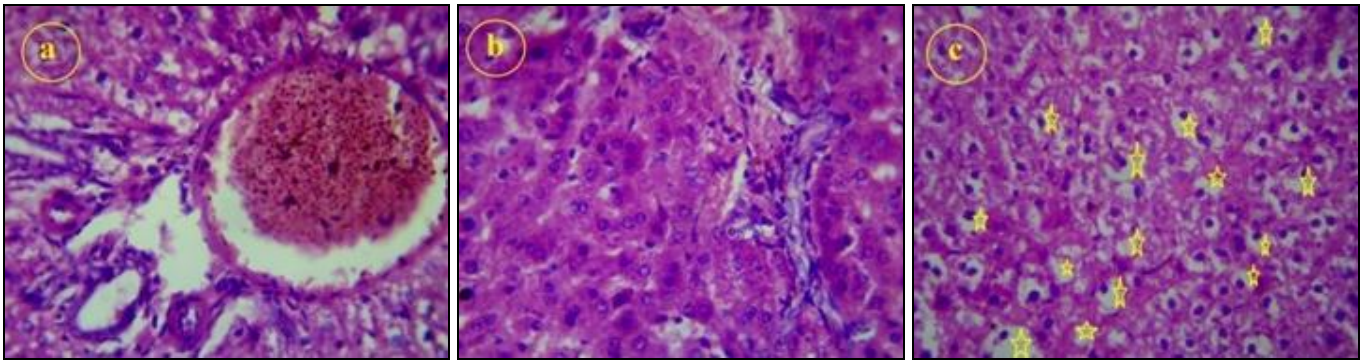

FIG. 7: HISTOPATHOLOGY OF LIVER

Fig. 7 (a-c) showed histopathology of liver under 40X magnification. Fig. 7a control rats showed normal liver cells. Fig. 7b Infected rats showed normal hepatocytes. Fig. 7c Linezolid treated infected rats showed macrovesicular steatosis (star).

Histopathological Results: Histopathological findings were shown in **Fig. 7**. Microscopic examination of control and infected rats showed normal hepatic architecture **Fig. 7a** and **b**. The LZD treated group revealed macrovesicular steatosis and apoptotic hepatocytes with diffuse vacuolization of hepatocytes **Fig. 7c**.

DISCUSSION: MRSA is a predominant pathogen for complicated skin and soft tissue infections (SSTIs). MRSA infections are characterized by liquefaction of infected tissue and abscess formation; the resulting cause's ischemia and necrosis. Hematogenous dissemination causes septicemia and spread to other organs pose higher risks of mortality and functional disability [13]. Linezolid is a better option to treat MRSA diabetic and orthopedic infections. Eradication of these infections requires long-term therapy of linezolid. In our study linezolid used for 2 weeks against MRSA infected rats. It causes severe hematological toxicity (decrease RBC, WBC, PLT, and Hb) are mainly due to myelosuppression, immune-mediated mechanism and oxidative stress have an essential role in the structural and functional damages of hemocytes [14]. Waldrep *et al.,* [15] demonstrated that linezolid treatment increases the iron saturation in the bone marrow and cause a reduction in RBC and Hb content. Antibiotics cause dysbiosis, an imbalance in the number and composition of intestinal bacteria, which facilitate the growth of opportunistic bacteria. Gut enzyme IAP Physiologically, exhibit many functions, including decreasing dietary fat absorption and detoxifying bacterial toxins.

In our study, the LZD treated rats showed a diminished level of IAP and caused alterations in the microbiome, intestinal inflammation, and intestinal permeability. The other orally entered bacterial endotoxins easily penetrate the intestine and increase the risk of bacterial disease.

LZD treated rats showed macrovesicular steatosis in liver cells due to an accumulation of fat and fatty degeneration, but no alteration in circulatory lipid levels. The LZD administration in infected rats showed elevated hepatic enzymes indicates hepatotoxicity.

Lactic acidosis is a common burden for intensivists. Nevertheless, clinical studies revealed the incidence and outcome of lactic acidosis are infrequent and are both retro and prospective with small sample size [16]. Lactic acidosis is a condition in which > 4-5 mmol/l of serum lactate causes metabolic acidosis. LZD inhibits bacterial protein synthesis by binding residues in the 23S ribosomal RNA of the 50S bacterial ribosomes. LZD binds to human ribosomes, leading to a decrease in the activity of respiratory chain complexes having mitochondrial DNA-encoded subunits and decrease the protein of the complexes, due to similarities between bacterial and human ribosomes.

The patient with LZD therapy decreased the mitochondrial enzyme activity in tissues. LZD is metabolized mainly by the liver, and the liver also metabolizes more than 60% of lactic acid. Therefore, liver dysfunction can be considered as a risk factor for LZD-induced lactic acidosis. In the case of patients with lactic acid, overload leads to death. The linezolid induced lactic acidosis due to mitochondrial DNA polymorphisms [17]. The deposition of lactic acid induced by LZD also damages to organs such as liver and kidney.

CONCLUSION: The present study concludes that linezolid has effectively acted against soft tissue MRSA infections. The prolonged use of linezolid causes intestinal dysbiosis, myelosuppression, mitochondrial toxicity, and hepatotoxicity. Toxicities produced by prolonged linezolid therapy must be tested against MRSA infected different diseased population.

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CONFLICT OF INTEREST: None

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