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# Identification of *mazEF* Toxin – Antitoxin system and biofilm formation in clinical isolates of MRSA isolated from Eastern India

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**Abstract:** *Introduction:* Interest on Toxin-Antitoxin (TA) systems has increased dramatically over recent years. It is ubiquitously present in many bacterial genomes including pathogens like MRSA. Several cellular functions of TA systems are proposed like programmed cell death, persister cell formation, biofilm formation etc. However, their exact role in cell physiology remains unclear. Due to the biofilm development the rate of morbidity and mortality of chronic MRSA infections are increasing day by day. *Objectives:* The aim of the study is to find out biofilm formation and *mazEF* toxin-antitoxin systems in clinical isolates of MRSA, isolated from tertiary care hospital, Kolkata. *Materials and Methods:* MRSA isolates were detected for biofilm formation by tube method and simultaneously the presence of *mazEF* TA gene in these MRSA isolates were evaluated. *Results:* It was found that 52.47 % MRSA isolates were potent biofilm producers by Tube method and 100 % of the MRSA isolates possessed *mazEF*gene. *Conclusions:* The analysis recommended that TA genes are highly prevalent in clinical isolates of MRSA strains. Further to come into any conclusion reverse transcription studies and TA mutants are needed to be constructed in order to find out the exact amount of *mazEF* expression in all MRSA isolates and rapid detection of biofilm producers is necessary as it may lead to antibiotic resistance & thereby difficulty in treating patients.

**Keywords:** Biofilm, MRSA, *mazEF* Toxin-Antitoxin Systems

## Introduction

In 1983, TA systems were discovered as plasmid addiction systems due to their ability to stabilize plasmids by post segregational killing in low-copy-number plasmids [1]. Toxin-antitoxin (TA) systems are pair of genes in an operon which encodes a stable toxin and its labile antitoxin that inhibits the action of the toxin. Several toxin-antitoxin modules have been identified in the chromosome of *E. coli.* Among them the *mazEF* system was the first to be described and studied [2].

*mazF* encodes the stable toxin and *mazE* encodes the labile antitoxin. MazF is an endoribonuclease that cleaves mRNAs at ACA sequences in a ribosome-independent manner [3-4]. MazE is degraded by the ATP-dependent ClpPA serine protease [2]. As long as MazE and MazF are co-expressed, MazE counteracts the toxic activity of MazF [2]. Since MazE is a labile protein, preventing MazF-mediated action requires the continuous production of MazE. Thus, in any stressful condition like antibiotics inhibiting transcription, translation or causing DNA damage prevents the expression of the chromosomally borne *mazEF* module which will lead to the reduction of MazE in the cell, permitting toxin MazF to act freely causing bacterial cell death [5-9].

It remains controversial whether *mazEF* mediated cell death involved in biofilm formation. It has been hypothesized that in a bacterial culture, the death of some of the cells may provide extra-cellular matrix molecules and nutrients to the remaining alive cells [10]. It seems likely that by increasing such molecules biofilm formation would be

supported. In this study we will try to detect the presence of *mazEF* genes in MRSA strains isolated from different clinical samples. Simultaneously we will evaluate the prevalence of biofilm forming MRSA isolates.

## Material and Methods

*Confirmation of MRSA isolates: Staphylococcus aureus* was isolated from various clinical samples like pus, sputum, urine, blood and other body fluids coming to Department of microbiology, Medical College, Kolkata for routine examination. The samples were initially streaked in Blood agar plates followed by 24 hrs incubation at 37°C. *Staphylococcus aureus* was confirmed by Gram staining, slide coagulase test, tube coagulase test and anaerobic mannitol fermentation by using standard methods [11].

The identities of *S. aureus* isolates were further confirmed by *16S rRNA* PCR. All the confirmed *Staphylococcus aureus* strains were subsequently tested for methicillin resistance by Kirby-Bauer disc diffusion method using cefoxitin discs (30 µg/disc) (Hi-Media, India) on Mueller-Hinton agar. The isolates were considered cefoxitin resistant if the zone of inhibition was 21mm or less. Results were interpreted according to CLSI guidelines (2014). Further *mec A* gene PCR, responsible for methicillin resistance was performed. The details of primers and PCR conditions are given in [Table 1].

**Table-1: PCR primers and conditions used in the study**

| Gene | Primer sequence | Product length (bp) | PCR Conditions | Reference |
|---|---|---|---|---|
| *16S rRNA* | (+)GTA GGT GGC AAG CGT TAT CC<br>(-)CGC ACA TCA GC GTC AG | 228 | 5min -94ºC-30sec<br>64ºC-30sec,72ºC-1min | [12] |
| *mecA* | (+)AGTTGTAGTTGTCGGGTTT<br>(-)AGTGGAACGAAGGTATCATC | 604 | 5min -94ºC-1min<br>54ºC-1.5min, 72ºC-1min | [13] |
| *mazEF* | (+)ATCATCGGATAAGTACGTCAGTTT<br>(−)AGAAGGATATTCACAAATGGCTGA | 408 | 5min -94ºC-45sec<br>54ºC-45sec, 72ºC-1min | [14] |

*Biofilm formation by Tube Method:* For biofilm detection we followed the tube method with slight modifications [15]. Isolated colonies of MRSA strains were inoculated in 10ml of trypticase soy broth (TSB) with 1% glucose and then it was incubated at 37ºC for 24 h. After incubation, the tubes were decanted,washed with 0.2 ml of phosphate buffer saline (pH 7.2) and dried. This removed free floating bacteria. The biofilm formed by bacteria, adherent to the tube walls were fixed by baking for 60 minutes and were then stained by crystal violet (CV) (0.1%).

Excess stain was removed by deionized water. Tubes were dried in inverted position. Then to dissolve the formed biofilm ring on the adherent tube walls 1ml dimethyl sulfoxide (DMSO) was added to each tubes. Vortex and finally optical density (OD) of stained adherent biofilm was obtained spectrophotometrically by using wavelength of 570 nm. The uninoculated tube containing only TSB were used as control. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al. [16] Positive and negative control were also inoculated.

*Detection of mazEF genes by PCR amplification:* Detection of Toxin-Antitoxin gene *mazEF* in MRSA strains was done using PCR technique with specifically designed primer pairs as mentioned in [Table 1]. DNA extraction was performed by boiling lysate method [17].

DNA amplification was performed in 25 µl of reaction mixture that contained 2.5 µl l0x buffer, 2.5 µl of 200mM dNTP mix (I.D.T), 2 µl of 25 mM of $MgCl_2$, 1.5µl of 10 pmol

each of a pair of primers forward and reverse (I.D.T) , 10 ng of staphylococcal template DNA and 1U of Ampli-Techgold Taq DNA polymerase. PCR amplification was carried out in a DNA thermal cycler (Applied Biosystem 2720) under reaction conditions as described in [Table 1].

The amplification product was resolved by electrophoresis on 1.5 % agarose gel at 70V (constant voltage) and visualized with ultraviolet light after staining with ethidium bromide. Product size was determined by using the 500bp DNA molecular weight ladder (Neb).

## Results

Samples received in microbiology laboratory of Medical College Hospital, Kolkata, West Bengal from various departments were processed for 1 year from August 2013 to July 2014. Bacterial pathogen was identified in 3002 samples of which 448 (14.92 %) were Gram positive strains.

Out of which 101 (22. 54 %) were MRSA isolates. From this 53 (52.47%) were biofilm formers and 47 (46.53%) were non biofilm producers. (Fig 1) Out of 101 isolates, all (i.e 100%) strains showed the presence of TA (*mazEF*) gene. (Fig 2).

**Fig-1:** The Biofilm Producers by Tube Method: A. High, B. Moderate and C. Non-Biofilm Producers detected by Crystal Violet Staining in glass tubes

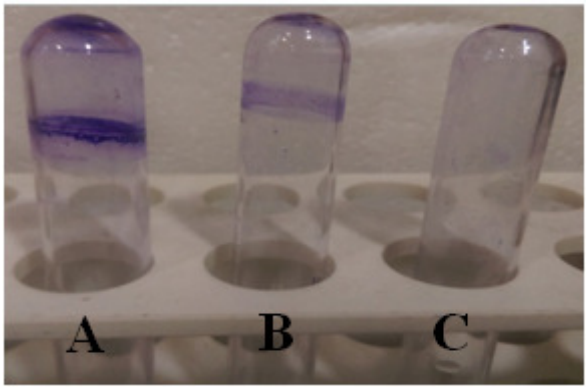
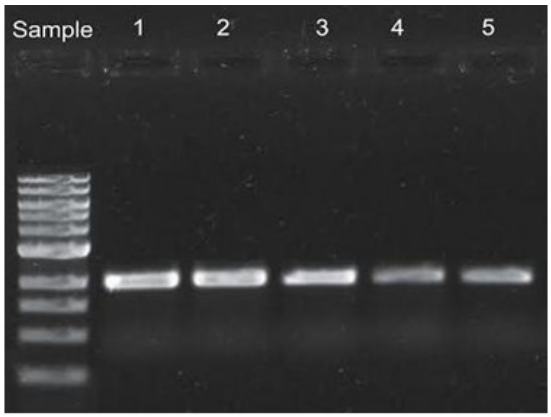



**Fig-2:** Agarose gel electrophoresis of PCR-amplified *mazEF* genes of clinical isolates.




Lane 1: 500 bp ladder; Lane 1-5: five clinical isolates positive for *mazEF* gene at 408bp.

## Discussion

Evidences are there that TA systems regulate genes of other operons, mediate the general stress response, and directly helps cells toward biofilm and persister cells formation. The MqsR/MqsA TA system of *E. coli* was found to be first TA pair linked to biofilm formation [18]. The importance of this TA system in biofilm formation was confirmed by another follow-up publication that linked it to motility and the quorum sensing system [19].

Recently these results linking MqsR/MqsA to biofilm formation were again confirmed using 48-h biofilms in which deletion of *mqsRA* reduces biofilm formation [20]. Further the role of TA systems in biofilm formation was obtained by studying a Δ5strain in whch five of the TA systems named MazF/MazE, RelE/RelB, YoeB/YefM, YafQ/DinJ and ChpB were deleted . Upon deletion of these five TA systems, biofilm formation decreased after 8 h and further increased after 24 h in rich medium at 37°C due to reduction in dispersal phenomena [21].

The results indicating that the TA systems of the Δ5 strain regulate biofilm formation were confirmed by another study. It was found that deletion of each same system independently decreased biofilm formation after 8 h and further increased after 24 h in rich medium due to decreased cell lysis [22]. But recently a study was published that demonstrates that

there is no significant association between biofilm formers and *mazEF, relBE, ccdAB, and mqsRA* positive isolates, except for the case of *hipBA* TA system [23]. The published prokaryotic genomes data demonstrated the universal nature of TA loci [24]. However, little effort has been made to survey large collections of clinical strains for the presence and functionality of TA systems. Herein by PCR technique we found that *mazEF* is highly prevalent in MRSA clinical isolates.

## Conclusion

These studies served to identify that *mazEF* TA systems are highly prevalent in clinical MRSA strains isolated from West Bengal and in future activation of TA systems could be used as an attractive antimicrobial strategy, since the freed toxin can kill the host bacterial cell.

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