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Analysis of expression profile of *mce* operon genes (*mce1*, *mce2*, *mce3* operon) in different *Mycobacterium tuberculosis* isolates at different growth phases

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***Background & objectives*: *Mycobacterium tuberculosis* (*M. tuberculosis*) has four homologous mammalian cell entry (*mce*) operons (*mce1-4*) that encode exported proteins and have a possible role in the virulence mechanism of this pathogen. The expression of *mce* operon is considered to be complex and not completely understood. Although expression of *mce* operon at different *in vitro* growth phases has been studied earlier, its expression in different *M. tuberculosis* isolates under different growth phases is not yet studied. The present preliminary study was conducted on a limited number of isolates to know the trend of expression pattern of *mce* operon genes in different *M. tuberculosis* isolates under different growth stages.**

***Methods*: In this study, we monitored the transcriptional profile of selected *mce* operon genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) in different *M.tuberculosis* isolates (MDR1, MDR2, and sensitive isolate) at early exponential and stationary phases using real-time quantitative PCR.**

***Results*: The expression ratio of all selected *mce* operon genes in all *M. tuberculosis* isolates was reduced at the initial phase and increased substantially at a later phase of growth. Higher expression of *mce1* operon genes was found in all *M. tuberculosis* isolates as compared to other *mce* operon genes (*mce2* and *mce3* operons) at stationary growth phase.**

***Interpretation & conclusions*: The higher expression of *mce* operon genes at stationary phase (as compared to early exponential phase) suggested growth phase dependent expression of *mce* operon genes. This indicated that the *mce* operon genes might have a role in *M. tuberculosis* survival and adaptation on the onset of adverse condition like stationary phase. Identification of differentially expressed genes will add to our understanding of the bacilli involved in adaptation to different growth conditions.**

Key words Drug resistance - gene expression - mammalian cell entry operon - *Mycobacterium tuberculosis* - real-time PCR

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The emergence of HIV-TB co-infection and multi-drug resistant cases of TB has increased the severity and magnitude of the TB epidemic. There is limited information about the behaviour and virulence factors of drug resistant isolates of *Mycobacterium tuberculosis* (*M. tuberculosis*). In the pathogenesis of *M. tuberculosis* the most critical step is the entry into the host cells (macrophages) and its intracellular growth and survival[1,2]. The survival of bacterium in host cells and also its adaptation to different physiological conditions (*in vivo* and *in vitro*) depend on expression of different sets of genes[3]. Therefore, study of *M. tuberculosis* genes responsible for bacilli adaptation to different physiological conditions (virulence property) would be of great importance to understand the pathogenesis of tuberculosis.

The mammalian cell entry (*mce*) genes encode for invasive/adhesive cell surface proteins and possibly have a role in invasion of host cells during the early events of infection and survival of *M. tuberculosis* in macrophages[4-8]. The whole genome sequencing of *M. tuberculosis* revealed the presence of four dispersed but homologous *mce* operons (*mce1*-4) arranged in a nearly identical manner and having two integral proteins *(yrb* EA-B) and six *mce* genes *(mceA-F)*[5,8,9]. The presence or absence of *mce* operon does not largely determine the pathogenicity[9-11], however, its characteristic expression may be of importance in the virulence. Earlier, inactivation of *mce2* operon in *M. bovis* BCG has been shown to reduce its ability to invade non-phagocytic HeLa cells indicating a role of *mce2* operon-encoded proteins in virulence[12]. *mce* operons are known to be expressed as polycistronic transcripts and their expression profiles vary in a growth phase specific manner both in liquid and solid culture[13,14] .

The genetic basis of drug resistance has largely been studied and candidate genes along with mutations have been identified[15]. However, it has also been found that a proportion of drug resistant isolates of *M. tuberculosis* do not have the expected mutations suggesting the involvement of some other mechanism(s) responsible for resistance[16,17]. Moreover, *M. tuberculosis* grows under various stress conditions within cells, and these conditions may provide a hypermutable background, making resistance more likely. The genetic variations in *mce* operon genes (*mce1* and *4*) have been studied in clinical isolates of *M. tuberculosis* and high level of polymorphism of operon genes has been reported in drug sensitive isolates as compared to resistant[18]. As the levels of expression of the *mce* operon genes in different *M. tuberculosis* isolates during different growth (*in vitro*) stages might be necessary for evaluating their putative functions, virulence mechanism, *etc*., therefore, we explored the *mce* operon genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) expression analysis in different *M. tuberculosis* isolates at early exponential and stationary growth phases (*in vitro*) by quantitative real-time PCR.

Material & Methods

Bacterial strains: This study was conducted in the Microbiology and Molecular Biology department of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJILOMD), Agra, India. A total of four *M. tuberculosis* isolates referred to as 'MDR1', 'MDR2', 'S' and a standard strain 'H37Rv' (TMC102, taken from our Institute repository) were included in the study. All isolates were confirmed as *M. tuberculosis* by standard biochemical tests[15], and were reconfirmed by PCR-RFLP (restriction fragment length polymorphism) detection based on primers targeting *hsp* 65 KD[19] and 1.8kb[20]. All *M. tuberculosis* isolates were subjected to drug susceptibility testing by standard L-J (Lowenstein-Jensen) proportion method[21] against four first-line antitubercular drugs [0.2 µg/ml of isoniazid (INH),4 µg/ml of streptomycin (STR), 40 µg/ml of rifampicin (RIF) and 2 µg/ml of ethambutol (EMB)]. Based on the drug susceptibility testing 'MDR1' was resistant to rifampicin, isoniazid, ethambutol and streptomycin whereas 'MDR2' was resistant to only rifampicin and isoniazid. Similar to standard H37Rv strain, 'S' was also susceptible to all four drugs, and was used as absolute control and expression of targeted operon genes in H37Rv was used to normalize the expression of the same genes in other three selected isolates of different drug sensitivity profile.

Culture and harvesting: All *M. tuberculosis* isolates were grown in Middlebrook 7H9 broth supplemented with albumin dextrose catalase (ADC supplement) (Difco, USA) and 1 per cent glycerol at 37°C as a primary culture. Using the 10 per cent of primary culture, secondary culture was cultivated into 500 ml screw cap conical flasks, each having 100 ml of medium in shaking condition. Cells were grown below an OD_{600} of 0.3. The bacilli were recovered at early exponential phase (OD_{600} of 0.8) and stationary phase (OD_{600} of 2.0) and centrifuged at 6000 *g* for 10 min at 4°C. The pellet was washed twice with chilled 1x phosphate buffer saline (PBS) before processing for RNA isolation.

RNA isolation and cDNA synthesis: Total RNA was obtained from growing cultures of *M. tuberculosis* isolates in early exponential and stationary phases of growth using commercially available Trizol reagent (Invitrogen, Darmstadt, Germany) as per manufacturer's instructions. From different growth phases, appropriate amount of culture was harvested in duplicate from three independent experiments. Complementary DNA (cDNA) was synthesized from isolated RNA in a reaction volume of 20 µl by using first strand cDNA synthesis kit (Fermentas Life sciences, Leon-Rot, Germany) using 500 ng of RNA, random hexamer (0.5 µg/µl) and avian myeloblastosis virus- reverse transcriptase (AMV-RT) (10 U/µl).

Quantitative real-time PCR and data analysis: A total of six genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) of *mce* 1, 2 and 3 operons were studied using self designed primers (using Primer 3 software, *www.frodo.wi.mit.edu/primer3/*) commercially procured from Sigma Aldrich, Bengaluru, India. The nucleotide sequences of each primer are given in the Table. Each 10 µl real-time PCR reaction in SYBR Green I format consisted of 5 µl of SYBR green mix, 0.5 µl forward primer (0.5 µM), 0.5 µl reverse primer (0.5 µM), 2 µl nuclease-free PCR grade water and 2 µl cDNA (0.5 µg) was added in a DEPC (diethylpyrocarbonate) treated microcentrifuge tube. The reaction mixture was further subjected to LightCycler 480II instrument (Roche Diagnostics, Mannheim, Germany) and fluorescent data were acquired during each extension phase. PCR cycling conditions were similar as 95°C, 10 min (95°C 10 sec, 62°C 15 sec, 72°C 12 sec) X 45 cycles 72°C, 2 min annealing temperature for each primer. In each set of reactions, fold changes were described in the form of threshold cycle (*C*t) and the transcript levels of the target genes of the three *M. tuberculosis* isolates were normalized with the reference gene 16S rRNA and then normalized to the values obtained from control strain H37Rv. To analyse the quantitative differences in gene expression in all the selected genes at both phases and also among different isolates of *M. tuberculosis* in comparison to control strain, logarithmic graphics of the fold change expression of each gene were created.

Table. Primers used for quantitative real-time PCR

Targeted genes	Forward and reverse primer sequences (5'–3')	Amplicon size (bp)
*Mce1*A	TCGCGCCACGACATTCAGCA TCCGCTTGCTGGGCATTGATGG	125
*Mce1*D	AAGATCGAACCAGCCGGCGA AATGTTCCGCGACGCCACCA	120
*Mce2*A	ATGTTCGGCGGTTGGCGGAT TCCAGATCGCGCTGTTGGCT	121
*Mce2*D	ACGCGAAGCCTGGCGCTATT TTCCACCGTGTCGGCCAAGT	132
*Mce3*A	TCGACCTGGTGATGCCGCAAAA AGCGGGTCGATCATGTTGAGCA	136
*Mce3*C	TGCGCGACACGAATGTGGTCT AGCGATGAAGCCCCGCAGTT	110
16S rRNA	GCGCAGATATCAGGAGGAAC AAGGAAGGAAACCCACACCT	150

The relative expression ratio was calculated from triplicate normalized ratios for each gene, and quantitative real-time PCR data were analyzed by using the $2^{-(\Delta\Delta Ct)}$ method as described by Livak and Schmittgen[22]. Relative expression (in fold change) of targeted gene of 'MDR 1', 'MDR 2' and 'S' was estimated using 16S rRNA gene as a control (for calculation of *Ct* value) and same gene of H37Rv strain as calibrator (for calculation of $\Delta\Delta Ct$). The logarithmic graphs depicting fold change in expression of each targeted gene of different isolates were prepared. The significant difference of gene expression during exponential and stationary phase was compared using student t test and significant differences in the expression of the gene in the isolate were analysed using one way ANOVA by Graph pad prism (Version 5) software (San Diego, California, USA).

Results

The relative expression of all selected *mce* operon genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) was significantly higher at stationary phase as compared to early exponential phase in all *M. tuberculosis* isolates (MDR1, MDR2 and sensitive isolates) (Fig. 1). We selected first (A) and middle genes (C, D) of each *mce* operon (*mce1*-3) as a representative of operon to evaluate their level of expression by quantitative methods.

The relative expression of *mce1* operon genes was significantly lower during early exponential phase (*mce1*A gene-0.394 ± 0.0403, 0.454± 0.031, 0.676 ± 0.029 and *mce1*D gene-0.543 ± 0.021, 0.521 ± 0.015, and 0.535 ± 0.060 in MDR1, MDR2, sensitive isolates, respectively) as compared to stationary phase (*mce1*A gene-262.9 ± 41.08, 21.8 ± 1.174, 1257 ± 227 fold and *mce1*D gene-4.48 ± 0.155, 1.93 ± 0.113, 46.3 ± 3.627 fold in MDR1, MDR2, sensitive isolates,

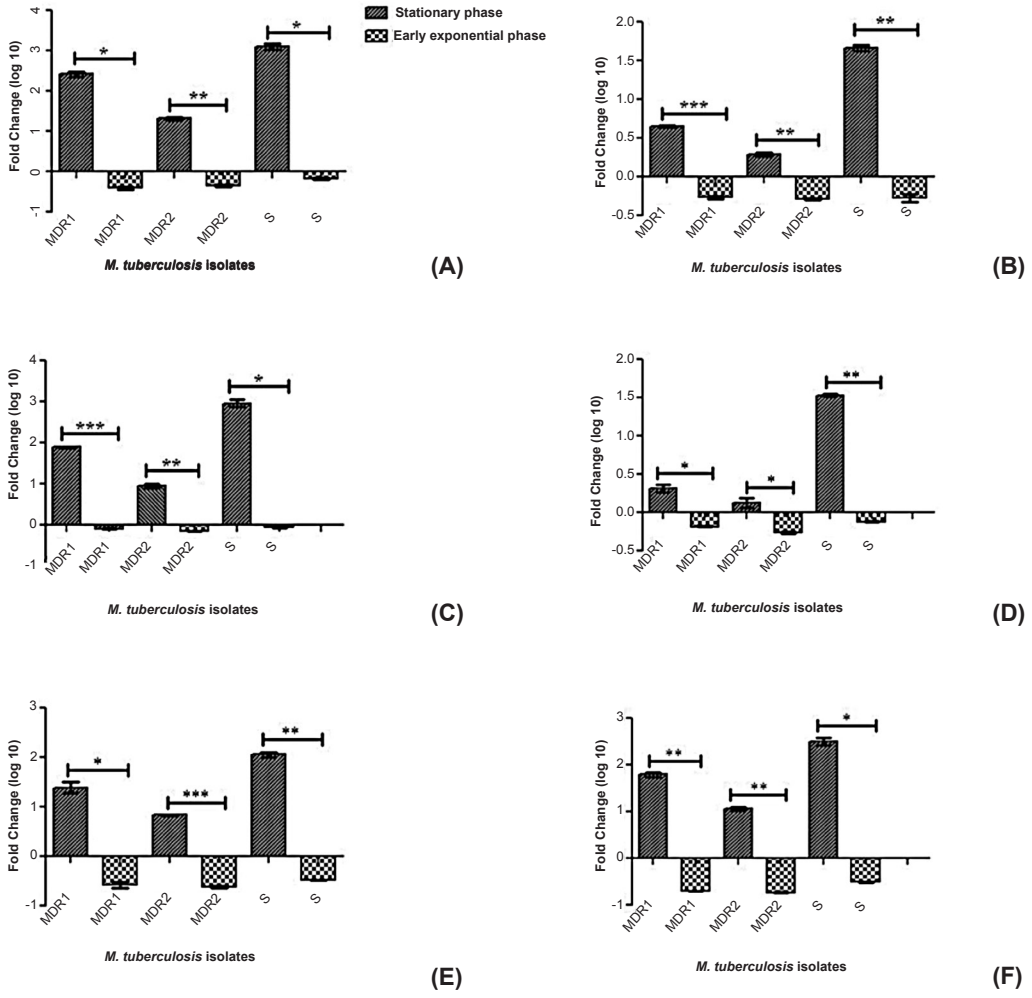

Fig.1. Relative expression (Fold change in logarithmic graphics) analysis of *mce1*A gene **(A)**, *mce1*D gene **(B)**, *mce2*A gene **(C)**, *mce2*D gene **(D)**, *mce3*A gene **(E)**, *mce3*C gene **(F)** of *M. tuberculosis* isolates (multi drug resistant and sensitive) during two different growth phases. The relative expression (analyzed by student t test) of all *mce* operon genes was significantly higher in stationary phase as compared to early exponential phase (P<0.001) for all the three isolates. MDR1-Multi-drug resistant isolate (resistant to rifampicin, isoniazid, ethambutol streptomycin), MDR 2- Multi drug resistant isolate (resistant to both isoniazid and rifampicin), S, sensitive isolate. P^{*}<0.05, **<0.01, ***<0.001. Values are mean ±SD of three experiments.

respectively) and both genes differed significantly for both phase in all the three isolates. (P<0.01, <0.01, <0.05, respectively). The relative expression of *mce2* operon genes was lower in early exponential phase (*mce2*A gene-0.824 ± 0.040, 0.729 ± 0.004, 0.882 ± 0.008 fold and *mce2*D gene- 0.659 ± 0.006, 0.547 ± 0.021, 0.760 ± 0.011 fold in MDR1, MDR2, sensitive isolates, respectively) compared to the stationary phase (*mce2*A gene -79.4 ± 3.111, 9.03 ± 1.146, 932.6 ± 190.9 fold and *mce2*D gene- 2.07± 0.247, 1.33 ± 0.190, 33.9 ± 1.499 fold in MDR1, MDR2, sensitive isolates, respectively) and both genes differed significantly for both phase in all the three isolates. (P<0.05 for all). The relative expression of *mce3* operon genes was lower in early exponential phase (*mce3*A gene-0.267 ± 0.0417, 0.240 ± 0.008, 0.333 ± 0.001 and *mce3*C gene-0.203 ± 0.0021, 0.185 ± 0.0035, 0.316 ± 0.0056 in MDR1, MDR2, sensitive isolates, respectively) as compared to stationary phase (*mce3*A-25.06 ± 6.20, 6.84 ± 0.0989, 115.3 ± 12.97 and *mce3*C gene-62.4 ± 6.718, 11.5 ± 1.188, 318.5 ± 54.39 in MDR1, MDR2, sensitive isolates, respectively) and both genes differed significantly for both phase in all the three isolates (P<0.05, <0.001 and <0.01, respectively).

The ANOVA analysis showed a significantly higher expression of all the studied genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) in stationary phase in all the three selected isolates (MDR1, MDR2, sensitive isolates) (P<0.001 for all the three isolates). Further, to analyse the significance of the genes of a particular isolate (MDR1, MDR2, sensitive isolate separately) one way ANOVA analysis was performed, which showed a significant difference in expression between the genes of the same operon and genes of different operon in a single isolate. The *mce1*A gene (*mce1* operon) showed a significantly higher expression in extracellular growth at stationary phase as compared to *mce2*A (P<0.001) and *mce3*A (P<0.001) in all the three *M. tuberculosis* isolates (MDR1, MDR2, sensitive isolates). The *mce3*C gene (*mce3* operon) showed a higher expression (non significant) in extracellular growth as compared to *mce1*D and *mce2*D genes at stationary growth phase in MDR1 and sensitive *M. tuberculosis* isolates. The ANOVA analysis showed a significantly lower expression of all the studied genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) in early exponential phase in all the three selected isolates (MDR1, MDR2, sensitive isolate) (P<0.001 for all the three isolates). Higher expression was observed for the first gene of each operon as compared to middle gene of the same operon (except *mce3* operon genes). There was also found higher expression of all the selected *mce* operon genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) in sensitive isolate among all *M. tuberculosis* isolates (Fig. 2).

Discussion

In this study, we analysed the relative expression of *mce* operon genes (*mce1*A, *mce1*D, *mce2*A, *mce2*D, *mce3*A, *mce3*C) in different *M. tuberculosis* isolates (MDR1, MDR2, sensitive isolates) at early exponential and stationary *in vitro* growth conditions. We used quantitative real-time PCR to detect the transcript level more accurately as compared to previous studies[3,14,23,24], where all the observations were relied mostly on the results of semi-quantitative RT-PCR and only expression of *mce* operon was described in terms of presence or absence of *mce* gene transcripts in *M. tuberculosis* H37Rv. Although the expression of all genes of *mce1* operon during *in vitro*-grown *M. tuberculosis* in Dubos broth media (during exponential phase) through RT-PCR was demonstrated by Harboe *et al*[23], but Kumar *et al*[13] could not detect the transcript of *mce1* in L-J slant at stationary growth phase of *M. tuberculosis*. This discrepancy may be due to higher inoculum size, use of different culture media as well shaking of culture broth. The impact of cultivation medium, its condition (shaking and standing), inoculum size and stress/stimulus has been reported earlier[14].

In general, at an early growth stage, the expression of all selected *mce* operon genes showed suppression but after that increased at stationary phase of growth. Transcriptionally and metabolically activeness of stationary phase bacilli was suggested by Voskuil *et al*[25]. The higher expression of *mce* operon genes may also be due to possible over-expression under nutritionally limiting conditions (such as the stationary phase of culture) to take up the available nutrients and also to effectively eliminate the secondary metabolites[14]. Kumar *et al*[13] have demonstrated that during simple ageing of logarithmically growing cultures (stress conditions), the metabolic activity of *M. tuberculosis* can be slowed down *in vitro*, and during these stress conditions bacilli have to be adapted to this situation by differential expression of different genes. Thus, our results indicate that *mce1*, *mce2*, *mce3* operon genes might have a role in *M. tuberculosis* survival and adaptation on the onset of adverse condition like nutritionally depleted condition at stationary phase.

In the present study, significant variation was observed in expression of six *mce* genes at stationary growth stage, suggesting the levels of expression of the different *mce* genes might be important for evaluating their putative function. There was also higher expression of *mce1*A in comparison to other genes of the same operon (*mce1*D) or genes of other operon (*mce2* and *mce3* operons), suggesting that *mce1* operon (specially *mce1*A gene) has major functional role extracellularly. A previous study also revealed that *mce1* operon has higher expression in the extracellular environment as compared to within macrophages[6]. Studies on *mce* operon showed a recombinant surface protein (*mce1*A) of *M. tuberculosis* conferred upon a non-pathogenic *Escherichia coli*, which had the ability to enter inside the mammalian cells (HeLa cells) and survival[4,7] suggesting roles of *mce1* operon both in cell entry and survival. Harboe *et al*[23] also demonstrated the expression of all six genes of *mce1* operon in *in vitro*-grown *M. tuberculosis* by RT-PCR.

The differential expression profile of *mce* operon genes at different growth phases in this study was expected as an earlier study also showed growth phase dependent expression pattern of *mce* operon[13].

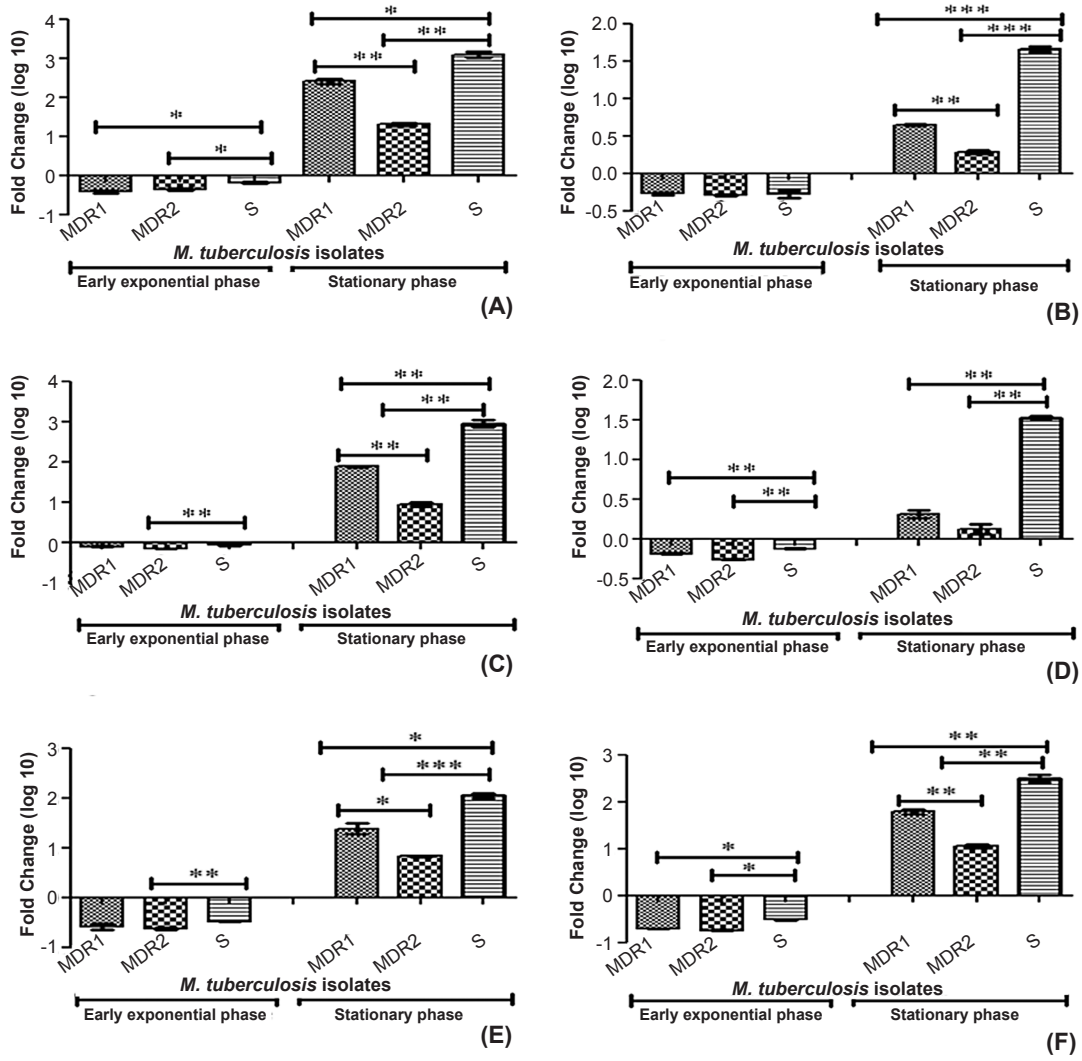

Fig. 2. Relative expression (fold change in logarithmic graphics) analysis of *mce1*A gene **(A)**, *mce1*D gene **(B)**, *mce2*A gene **(C)**, *mce2*D gene **(D)**, *mce3*A gene **(E)**, *mce3*C gene **(F)** of *M. tuberculosis* isolates (multi-drug resistant and sensitive) during two different growth stages (early-exponential and stationary phases) using gene specific primers by real time PCR. MDR1-Multi-drug resistant isolate (resistant to rifampicin, isoniazid, ethambutol), streptomycin, MDR 2- multi-drug resistant isolate (resistant to both isoniazid and rifampicin), S, sensitive isolate. $P^{*}<0.05$, $^{**}<0.01$, $^{***}<0.001$.

At stationary growth phase, the relative expression of all *mce* operon (*mce1*, *mce2*, *mce3*) genes remained higher for sensitive isolate among all *M. tuberculosis* isolates. The significant difference was observed in the expression profile of different *mce* operon in all the three *M.tuberculosis* isolates (MDR1, MDR2, sensitive isolates) at both growth phases, indicating complex regulation of *mce* operon genes expression. Pasrich *et al*[18] investigated the extent of polymorphism in eight genes in the *mce1* and 4 operon in 112 clinical isolates of *M. tuberculosis* varying in their drug susceptible profile (more polymorphic mutations in *mce* genes in the drug sensitive isolate as compared to resistant isolates). Since drug resistance provides an extra edge to resistant isolates, the more expression (as in present investigation) or polymorphic mutation[18] of *mce* operon genes in susceptible isolates may also relate to virulence mechanisms after adaptability to the environment. It was further supported by Shimono *et al*[26] who demonstrated the transformation of wild type *M. tuberculosis* strain into hypervirulent by disruption of *mce1* operon.

In the present study, relative expression of the *mce3*A gene was lower at both early exponential and stationary phases as compared to *mce1*A and *mce2*A genes. This may be due to dominant role of *mce1*A and *mce2*A genes (over *mce3A*) in *in vitro* growth and stationary phase adaptation. The *mce3*C gene (*mce3* operon) showed a higher expression (non significant) in extracellular growth as compared to *mce1*D and *mce2*D genes at stationary growth phase of bacilli in MDR1 and sensitive *M. tuberculosis* isolates. Overall, *mce 1* operon showed significantly higher expression in extracellular growth as compared to other *mce* operons (*mce2* and *mce3*). There was also higher expression for the first gene of each operon as compared to middle gene of the same operon (except *mce3* operon genes). With respect to expression of two genes of the same operon, no marked variation was observed for the selected genes of *mce1*, 2 and 3 operons at early exponential phase. At the stationary growth stage different mRNA transcript levels were measured for the same two genes of each *mce1*, 2 and 3 operons. Though our experiment resulted into an unexpected expression pattern of genes of the same *mce* operon but all genes of any operon will express similarly, is not necessary due to varying stability of different segments of mRNA and also because some operons have internal promoters or differential regulation of mRNA stability[27]. In *M. tuberculosis* system the effect of two promoters on regulation of an operon has been demonstrated earlier that resulted into different transcripts[28].

Joon *et al*[29] reported the presence of two functional promoters for *mce1* operon in *M. tuberculosis* that could potentially segregate different functions of a single operon. Casali *et al*[6] also showed that the expression of *mce* operon might be tuned under multiple negative regulators (*mce1*R, negative transcriptional regulator of *mce1* operon). It has been reported that *Mce3*R (a TetR family transcriptional regulator), downregulates the expression of *mce3* operon of *M. tuberculosis in vitro*[30]. Santangelo *et al*[31] showed that the expression of *mce3* operon of *M. tuberculosis* was regulated by *Mce3*R together with two transcriptional units, indicating a functional relation between the products encoded in the three operons. A previous study suggested differential expression of *mce* operon under different environmental and experimental conditions[13]. These findings suggest that *mce* operon regulation for *M. tuberculosis* may be more complex than one would expect for a prokaryotic system. Therefore, genes of the same *mce* operon, probably under the control of different regulators can express differentially.

In conclusion, the differential expression of *mce* operon genes at two different growth stages suggested growth phase dependent expression of *mce* genes, serving in adaptation of bacilli in different environmental conditions. The differences in expression of *mce* operon genes among *M. tuberculosis* isolates also indicated the possible association of *mce* genes expression with genetic context of the isolates (including drug resistance profile). Variation in expression of different *mce* operons at different growth phases is thought to be essential for bacterial survival and also variation in *mce* operon gene expression among *M. tuberculosis* clinical isolates has implications for pathogencity. Identification of such differentially expressed *mce* genes may be important for drug targets, vaccine antigens. The expression analysis of *mce* operon genes in different *M.tuberculosis* isolates at different growth stages may enrich our understanding about its role in its virulence.

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***Conflicts of Interest*:** None.

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