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Study on Survival of *Chlamydia trachomatis* in the Presence of Antichlamydial Drugs

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Abstract: Problem statement: Recurrent genital *Chlamydia trachomatis* infections due to treatment failures may results in complex sequelae leading to reproductive complexity and morbidity. It can be resulted by the heterotypic resistance with decreased drug susceptibility characteristic of the isolate. Studies are needed to understand the treatment failures and resistance characteristic of *C. trachomatis*. Hence, *in vitro* study was conducted on *C. trachomatis* isolate in the presence of antichlamydial drugs. **Approach:** Our aim was to study *ygeD* gene in *C. trachomatis* clinical isolate having decreased drug susceptibility profile and to analyze HeLa cells phenotypically upon infection in presence of antichlamydial drugs. Sequencing was done to check any mutational change (s) in *ygeD* gene of *C. trachomatis* isolate (CT-244), mRNA expression was analyzed in presence of antichlamydial drugs by Real Time RT-PCR. Transduction study was carried out in infected HeLa cells to detect changes at cellular level in presence of antichlamydial drugs by transducing with GFP/RFP-tagged proteins and analyzed by FACS. **Results:** A point mutation was detected in *ygeD* gene of *C. trachomatis* isolate. Further, mRNA expression level of *ygeD* gene was observed to be increased at 8 hpi in presence of doxycycline while in presence of azithromycin it was increased at 24 hpi. GFP-tagged plasma membrane protein expression in infected HeLa cells found to be reduced as compare to the uninfected cells. Upon infection, the RFP-tagged actin protein expression was up-regulated in comparison to the uninfected HeLa cells. No difference in expression of plasma membrane and actin protein was observed in susceptible serovar D and CT-244 isolate. **Conclusion:** The present study suggest that *C. trachomatis* isolate with decreased drug susceptibility profile may have an active efflux strategy for its survival in the presence of antichlamydial drugs and it may not affect its host cell plasma membrane or actin organization for its survival in order to resist the antichlamydial drugs.

Key words: *Chlamydia trachomatis*, recurrent infection, antimicrobial susceptibility, antichlamydial drugs, pelvic inflammatory, chlamydial infections, decreased susceptibility, protein expression

INTRODUCTION

Chlamydia trachomatis an obligatory intracellular pathogen causes a spectrum of clinically important chronic inflammatory diseases of human. *C. trachomatis* infection is one of the most prevalent sexually transmitted diseases in the world (Gerbase *et al*., 1998; Beagley and Timms, 2000). In females, *C. trachomatis* causes cervicitis, urethritis, ectopic pregnancy, pelvic inflammatory disease, tubal factor infertility and chronic pelvic pain (Morre *et al*., 2000). Studies have also implicated association of *C. trachomatis* infection with cervical and ovarian cancer and increase in HIV infectivity (Luostarinen *et al*., 2004). Antibiotics have the major role in treating chlamydial infections; azithromycin and doxycycline are considered as first line drugs by the Centers for Disease control and prevention (CDC) (Workowski and Berman, 2010). Efficacy of these drugs for treatment of chlamydial infections are high, however many researchers report the problem of recurrent infections and treatment failures Wang *et al*. (2005). It has also been reported that in women with persistent or recurrent infections, the infection can spread upwards from the endocervix to the fallopian tubes and may result in infertility or ectopic pregnancy (Hillis *et al*., 1997). Recurrent *C. trachomatis* infections often results from failure of antibiotic therapy or from reinfection due to

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unprotected sexual contact with either an untreated existing partner or a new infected Partner (Hillis *et al*., 1994). However, *C. trachomatis* atypical intracellular characteristics as persistent bodies are suggested to have a role resulting in refractory to antichlamydial drugs and recurrent infections (Beatty *et al*., 1994). Further, the emerging antibiotic resistance in chlamydia may create severe problems in the treatment of disease. There are few documented in vitro reports of antibiotic resistance in chlamydia but no examples of natural and stable antibiotic resistant strains collected from humans. Samra *et al*. (2001) few studies with clinical isolates of *C. trachomatis* from treatment failure patients demonstrated *in vitro* heterotypic resistance. Recently, 4 clinical isolates demonstrated *in vitro* resistance to macrolides were shown to carry mutations in the 23S rRNA gene (Misyurina *et al*., 2004). *In vitro* studies suggest that antibiotic-resistant genotypes of *C. trachomatis* can be generated and transferred to *C. trachomatis*, *C. suis* or *C. muridarum* isolates with capability of expressing significant resistant phenotypes (Sandoz and Rockey, 2011). Hence, emerging heterotypic bacterial resistance against antichlamydial drugs resulting in treatment failures in clinical settings cannot be neglected. Studies are needed for characterization of *C. trachomatis* clinical isolates showing decreased susceptibility towards the antichlamydial drugs which may results in resistant characteristics of the bacteria and can be concluded with respect to the patient's treatment failure (s) or reinfection (s).

Further, it has also been suggested that genotypic changes may not be only responsible for the resistant characteristics of clinical *C. trachomatis* isolate (s) obtained from multiple treatment failure patients. Different drugs have different targets for their action in bacteria hence; mutation in a single gene may not be suggested to result in multiple treatment failures. It has been reported that in gram-negative pathogens, efflux is the predominant mechanism of tetracycline resistance including *Chlamydia suis* Dugan *et al*. (2007). Hence, studies are needed to explore the role of efflux gene(s) in emerging resistance in *C. trachomatis*. In addition, in the presence of stress conditions host cell might play a role in altered drug sensitivity profile of bacteria. Resistant bacteria may act on various system of a cell directly or indirectly for its survival in the presence of drugs. According to many studies *C. trachomatis* changes host cell plasma membrane and actin organization by modifying its arrangements to complete its life cycle (Kumar and Valdivia, 2008a; 2008b).

In India, a high prevalence (>30%) of *C. trachomatis* infections in symptomatic female patients have been reported. (Singh *et al*., 2003) In our previous study the antibiotic susceptibility profile was studied towards the first line antichlamydial drugs and decreased *in vitro* susceptibility was observed in isolates (Bhengraj *et al*., 2010). Few of them appeared as of heterotypic resistant isolates in cell culture in the presence of antichlamydial drugs. Further we characterized them for presence of possible mutational changes at the reported resistant marker genes (L4, L22, 23SrRNA) (Bhengraj *et al*., 2011). However, genotypic characterization did not revealed any mutational changes at the drug target site(s), hence further characterization is needed.

Thus the aim was to study the efflux (*ygeD*) gene in *C. trachomatis* heterotypic resistant isolate for presence of any mutational change(s) and mRNA expression in cell culture condition in the presence of azithromycin and doxycycline. In addition to that host HeLa cell plasma membrane and actin was also studied to know if *C. trachomatis* indirectly affects on it in the presence of antichlamydial drugs to complete its life cycle, which may result *in vitro* altered drug susceptibility characteristics.

MATERIALS AND METHODS

Cell culture and Propagation of *C. trachomatis* clinical isolate: Human cervical epithelial adenocarcinoma cell line HeLa-229 was procured from NATIONAL CENTRE FOR CELL SCIENCES (NCCS), Pune, India. Cells were maintained and cultured in Eagle's Minimum Essential Medium (EMEM; HiMedia, India) as described previously (Bhengraj *et al*., 2010). *C. trachomatis* isolate (CT-244) was propagated in DEAE-dextran (30µg mL)$^{-1}$ treated HeLa cells in Dulbecco's Modified Eagle's Medium (DMEM; HiMedia, India) supplemented with 5% Fetal Calf Serum (FCS), 10µg mL^{-1} gentamicin, 1µg mL^{-1} amphotericin B, 1µg mL^{-1} cycloheximide at 35ºC with 5% CO_2, purified and stored at -80ºC in SUCROSE PHOSPHATE GLUTAMATE Medium (SPG, pH 7.0). Infectious titers were determined by titration on HeLa cell monolayers as described earlier (Bhengraj *et al*., 2010).

Antimicrobial agents: Azithromycin and doxycycline (Sigma-Aldrich) were dissolved according to the manufacturer's instructions and dilutions were prepared in DMEM cell culture medium without antibiotics.

DNA Isolation and Polymerase Chain Reaction (PCR): HeLa cells infected with *C. trachomatis* isolate were subjected to DNA extraction using QIAamp Viral RNA mini Kit (Qiagen, CA, USA) according to manufacturer's instructions. Briefly infected cells were harvested at 48 h. post infection (hpi) and the cell suspension was centrifuged at 3000 rpm for 10 min at 4ºC. Supernatants were centrifuged at 16000 rpm for

1hr at 4ºC; pellets were collected and processed for DNA isolation. Concentration of DNA was quantified spectrophotometrically at 260 nm (Biometra, USA). The amplification of efflux gene was carried out by POLYMERASE CHAIN REACTION (PCR) in a DNA Eppendorf Mastercycler personal Thermal Cycler (Eppendorf GmbH, Germany). The primer sequences are 5' ACGATCTTTCCGTGCATTGGTCGT3' for forward primer and 5'GCCATGTAAGAGCCGACACCCA3' for reverse primer (MWG-Biotech, Germany). The thermal conditions for amplification were initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 30s, primer annealing at 60°C for 1min and extension at 72°C for 2 min, then a final extension at 72°C for 10 min. The PCR product was visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide on Alpha Imager gel documentation system (AlphaInnotech, San Leandro, USA).

DNA sequencing*:* The PCR products were purified using Qiagen gel extraction kit as per manufacturer's instructions. The sequencing of purified PCR products was carried out using BigDye terminator v3.1 (Applied Biosystems, CA, USA) as per recommendations. Briefly, 75-150 ng μL^{-1} of purified PCR product and sequencing primers (1 pmol/µL) were added to 4 µL Big Dye Terminator Reaction mix and final volume was made up to 10 µL with autoclaved MilliQ water. Sequencing PCR was set up with 30 cycles of 30 sec denaturation at 96°C, 30 sec annealing at 55°C and 4 min extension at 60°C. After sequencing PCR, the products were purified and re-suspended in Hi-Di formamide (Applied Biosystems). The samples were denatured at 94°C for 5 min followed by a brief incubation on ice and loaded on the 3130XL Genetic Analyzer (Applied Biosystems). Sequence analysis was carried out using Sequence Analysis software (Applied Biosystems) and SeqMan module of DNASTAR v5.07 software.

RNA isolation and real-time RT-PCR analysis: HeLa cells monolayers were prepared by seeding (3×10^5 cells/well) in six-well tissue culture plates and infected with *C. trachomatis* inoculum at MoI of 2 as described earlier Bhengraj *et al*. (2010). Dilutions of drugs (0.5, 5 and 10 µg $mL)^{-1}$ were added at 2 hpi and cultures were incubated at 35°C in 5% CO_2. Total RNA was isolated at 8, 24 and 48 hpi using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions and quantified using a UV-VIS spectrophotometer. RNA was treated with DNase I to prevent DNA carryover. The isolated RNA was further tested by PCR to check any carryover DNA contamination. There was no amplification of product detected and the RNA was considered as DNA free. Complementary DNA was prepared using SuperScript™ First-Strand Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Real-time PCR was performed with the DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). The primer sequences used for efflux (*ygeD*) gene were F 5'ACGATCTTTCCGTGCATTGGTCGT3 and R 5' GCCATGTAAGAGCCGACACCCA3' and for endogenous control (16S rRNA) gene were 5'CTGCAGCCTCCGTAGAGTCTGGGCAGTGTC3'a nd 5'TTCAGATTGAACGCTGGCGGCGTGGATG 3' as described earlier. Mpiga and Ravaoarinoro (2006) Primers were of HPLC-purified grade and were commercially synthesized (MWG-Biotech AG, Ebersberg, Germany). The negative control consisted of nuclease free water substituted for cDNA. PCR amplification was performed in an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, CA, USA). For data analysis, the $2^{-\Delta\Delta Ct}$ method was used to calculate fold change (Livak and Schmittgen, 2001).

Transduction of HeLa cel: For targeting host cell actin and plasma membrane proteins HeLa cells were transduced with Cellular-Lights and Organelle-Lights transduction reagents (Molecular probes, Invitrogen, Carlsbad, CA, USA) respectively. The transduction was based on the BacMam technology of viral delivery for specific expression of a targeted (fluorescent) protein in mammalian cell.

Transduction was carried out according to the manufacturer's instructions. Hela cells ~1×10^6-4×10^6 were seeded in 50 cm^2 tissue culture Flask (Greiner, Germany), allowed to adhere and grow for approximately 24 h at 37°C, 5% CO_2. Cellular-Lights transduction solution was prepared in Dulbecco's Phosphate Buffered Saline (D-PBS) without Ca_2+ or Mg_2+. Upon reaching 70-80% confluency of the adhered cells, culture medium was aspirated and 5.5mL of the diluted transduction solution were added. The cells were incubated at room temperature (20-25°C) in the dark for 4 h with gentle rocking. Transduction solutions from the culture flask were again aspirated and culture medium without serum plus 1X enhancer were added. Cells were incubated for 2 h at 37°C and 5% CO_2. After incubation enhancer solution from the culture flask were replaced with the appropriate culture medium and incubated at 37°C, 5% CO_2 for >16 h. Same method was followed for transduction of HeLa cells with organelle-lights.

Transduced HeLa cells were plated in 6-well tissue culture plates with cell density of 3×10^5 cells/well in EMEM containing 10% FCS. On reaching the sub-confluence, monolayer were washed twice with PBS and infected with chlamydial EBs at MoI of 2. For

homogenous infection tissue culture plates were placed on a rocker for 2 h at 35°C after addition of serum free media containing EBs. Media containing unbound EBs were aspirated and supplemented with complete DMEM containing 10% FCS. Infected HeLa cells were incubated at 35°C with 5% CO_2. Thereafter at 2 hpi media was aspirated and replaced with fresh media containing azithromycin or doxycycline. After 48 hpi cells were analysed for fluorescence using flow cytometer (BD FACS Caliber) in FL-1 and FL-2 channel. For negating auto-fluorescence same pool of untransduced cells were used and appropriate setting was used for further acquisition and analysis. Flow histogram was analysed for geometric mean using FCS V3 express (DeNovo Inc). Every experiment was done in triplicate.

Statistical analysis: Differences between two groups were evaluated using Student t test and $p<0.05$ was considered significant.

RESULTS

***C. trachomatis* ygeD gene:** *C. trachomatis* efflux (*ygeD*) gene was checked for any changes in the genetic level. A fragment of 822bp was amplified and single band was observed in 1.5% agarose gel. Product was sequenced in both the directions and reviewed by assembling into alignments using reference sequence *C. trachomatis* serotype D (GenBank accession numbers NC000117). The sequence showed variation with a point mutation T to G in 734318 position of the studied *C. trachomatis* clinical isolate (CT-244).

Real-time RT-PCR analysis: *C. trachomatis* isolate (CT-244) efflux *ygeD* gene was studied for any changes in gene expression in the presence of doxycycline and azithromycin in host HeLa cells.

Increased expression of *ygeD* gene was detected at 8, 24 and 48 hpi in the absence of doxycycline. However, in the presence of doxycycline significantly ($p<0.05$) increased expression was observed only at 8 hpi while at 24 and 48 hpi it was found to be decreased in presence of all three concentrations of doxycycline (Fig. 1). On addition of azithromycin, no significant changes were detected at 8 and 48 hpi with all the three concentrations of drug however, at 24 hpi expression was observed to be significantly ($p<0.05$) increased (Fig. 2).

Host cell analysis: Upon infection with serovar D and CT-244 isolate expression of Green Fluorescent Protein (GFP)-tagged plasma membrane protein in HeLa cells were found to be significantly reduced as compare to the uninfected transduced cells.

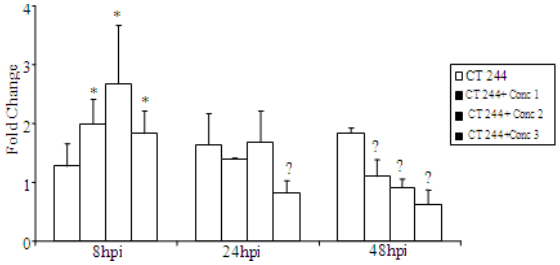

Fig. 1: Expression of *C. trachomatis* isolate (CT-244) efflux *ygeD* gene in HeLa 229 cells in the presence of doxycycline at 8, 24 and 48hpi. The graph showing results as fold change in expression of *ygeD* gene in CT-244 as compare to expression in the sensitive isolate by real time RT-PCR. * represents $p<0.05$ as compared to ''CT 244'' at 8 hpi, ♦ represents $p<0.05$ as compared to ''CT 244'' at 24 hpi and ▪ represents $p<0.05$ as compared to ''CT 244'' at 48 hpi. Conc 1, 2 and 3 represents 0.5, 5 and 10 μg mL^{-1} of doxycycline respectively. hpi = hour post infection

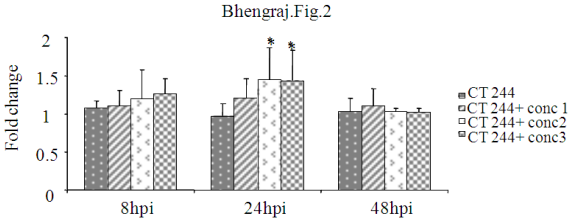

Fig. 2: Expression of *C. trachomatis* isolate (CT-244) efflux *ygeD* gene in HeLa 229 cells in the presence of azithromycin at 8, 24 and 48hpi. The graph showing results as fold change in expression of *ygeD* gene in CT-244 as compare to expression in the sensitive isolate by real time RT-PCR. * represents $p<0.05$ as compared to ''CT-244'' at 24 hpi. Conc 1, 2 and 3 represents 0.5, 5 and 10 μg mL^{-1} of azithromycin respectively. hpi = hour post infection

On addition of drugs, expression was found to be up-regulated in comparison to the absence of drugs and comparable to the uninfected cells. Further no difference in expression was observed in serovar D and CT-244 isolate in the absence of antichlamydial drugs. However at higher concentration of azithromycin and lower concentration of doxycycline non-significant difference was observed in the protein expression (Fig. 3).

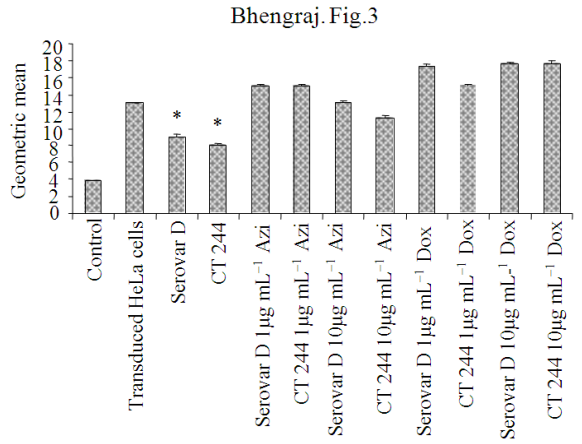

Fig. 3: Flow cytometric quantification showing geometric mean of expression of GFP-tagged plasma membrane protein in host HeLa cells harbouring *C. trachomatis* isolate (CT-244) in the presence of antichlamydial drugs (Azi: azithromycin and Dox: doxycycline), $p<0.05$ was considered as significant

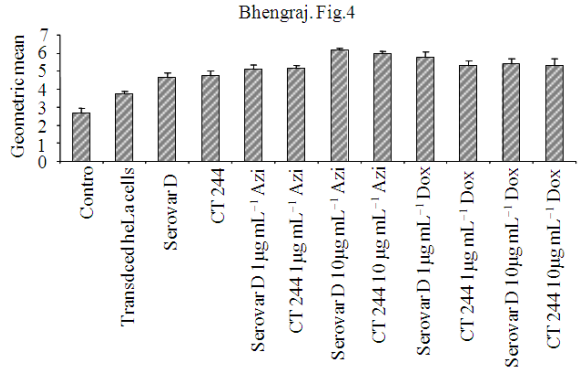

Fig. 4: Flow cytometric quantification showing geometric mean of expression of RFP-tagged actin protein in host HeLa cells harbouring *C. trachomatis* isolate (CT-244) in the presence of antichlamydial drugs (Azi: azithromycin and Dox: doxycycline)

Host cells were further studied for any changes in actin protein expression in the presence of antichlamydial drugs. The red fluorescent protein (RFP)-tagged actin protein expression was found to be up-regulated on infection with isolates in comparison to the uninfected HeLa cells. No difference in expression of actin was observed in serovar D and CT-244 isolate, however, addition of drugs increased the expression of RFP-tagged proteins in infected transduced cells (Fig. 4).

DISCUSSION

To avoid the severe sequelae of *C. trachomatis* infection, antibiotic strategies are important to eradicate the pathogen. First-line antichlamydial drugs have proven successful for the treatment of *C. trachomatis* infection however, treatment failures have been observed in notable number of cases Horner (2006). Few studies suggest resistance as a cause for clinical treatment failures (Jones *et al*., 1990; Somani *et al*., 2000). The obligate intracellular nature of *Chlamydia* may limit the emergence of antibiotic resistance *in vivo* (Abdelrahman and Belland, 2005). However, the extensive use of drugs has been known to favor the selection of resistance in pathogens, including *Chlamydia suis* in pig (Lefevre and Lepargneur, 1998). The role of genetic resistance in the recurrence of chlamydial infections is still not clear and needs further attention.

Besides well known mechanisms, a further resistance mechanism, active drug efflux, has become increasingly important in the current threat of multidrug resistance. It involves certain bacterial transport proteins which pump out antimicrobial compounds from the cell as a result of over expression of these pumps due to mutations hence decreasing intracellular antibiotic concentration. Efflux pumps possessed by various pathogens are likely to contribute their pathogenic mechanisms by escaping a number of antimicrobial compounds (Poole, 2005). Hence, we studied the efflux *ygeD* gene of heterotypic resistant *C. trachomatis* isolate in order to explore the resistant characteristics. The studied sequence showed variation with a point mutation T to G with reference sequence of serotype D. There was no difference in the products of the mutated nucleotide as reference sequence has CTT-Leucine amino acid and mutated nucleotide has CTG which also code for the leucine. This may be a non-significant mutation in developing in vitro resistance. In another study of efflux (*ygeD*) gene in clinical isolates of *C. trachomatis*- resistant to high and intermediate level of FQ concentrations several silent mutations and mutations resulting in amino acid substitutions were observed (Misiurina *et al*., 2004). Hence, this can be concluded that the mutation may not be directly related to the resistant characteristic of the bacteria but it might be possible that it has some indirect role, which may make bacteria more refractory to the drugs. Further expression of the efflux gene was also analyzed in the isolate and it was observed that efflux gene was actively expressed at 8hpi in presence of doxycycline, suggesting its expression may have helped in reducing doxycycline pressure at the initial time point. On

addition of azithromycin, expression of *ygeD* gene was observed to be significantly increased at 24 hpi suggesting that in the presence of azithromycin efflux gene was capable in reducing the drug pressure at 24 hpi but not at the initial time point. Hence, we may conclude that *C. trachomatis* isolate with altered drug susceptibility profile may have an active efflux strategy for its survival in the presence of antichlamydial drugs.

Antimicrobial susceptibility profile of *C. trachomatis* may be dependent on the host cell environmental conditions and host cell-specific factors. It is reported that oxygen concentrations in female urogenital tract affects the removal of chlamydia upon antibiotic treatment (Shima *et al*., 2010). In addition it has been observed that pathogenic microbes exploit the host cytoskeleton for entry, colonization and intracellular survival in eukaryotic cells (Rottner *et al*., 2005). *C. trachomatis* also co-opts host actin and intermediate filaments to form a dynamic scaffold for providing structural integrity to the chlamydial vacuole and minimizing immune detection for its survival. (Kumar and Valdivia, 2008a; 2008b) Hence, host cell factors should be studied to know if this affects the antibiotic susceptibility profile

Therefore, host HeLa cells harbouring the heterotypic resistant *C. trachomatis* isolate were studied for any phenotypic changes at the cellular level. The significantly reduced expression of GFP-tagged plasma membrane protein in HeLa cells detected may be due to the use of proteins for the invagination of infectious elementary bodies of *C. trachomatis*. However, on addition of drugs expression was found to be comparable to the uninfected cells. Further for detecting any changes in actin protein expression in the presence of antichlamydial drugs host cells were studied for RFP-tagged actin protein expression and it was found to be up-regulated upon infection. However, addition of drugs increased the expression in infected cells. There is no difference observed in expression of plasma membrane and actin protein in between the serovar D and CT-244 isolate. Hence, this may be suggested that *C. trachomatis* isolate with altered drug susceptibility profile do not affect its host cell plasma membrane or actin organization for its survival in order to resist the antichlamydial drugs.

CONCLUSION

In conclusion, our study supports the emergence of clinical antibiotic resistance, not an impossible scenario for *C. trachomatis* despite their isolated niche which limits the opportunity for acquisition of antibiotic resistance genes from other organisms (McOrist, 2000). Successful treatment is necessary for preventing sequelae of chlamydial infections hence, treatment failures and in vitro antibiotic resistance characteristics of *C. trachomatis* is of great concern. The results of present study in characterizing resistance in clinical isolate may enhance the understanding of chlamydial therapy and the nature or transmission of resistant *C. trachomatis*. Further, studies are needed in more number of *C. trachomatis* clinical isolates to know its biological relevance to *in vivo* conditions.

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