Existence of plasmid-less clinical isolate of Chlamydia trachomatis in India is a cause for concern and demands the use of real-time PCR assays

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Citation

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Abstract

Reports of Chlamydia trachomatis (CT) plasmid-less isolates worldwide have caused concern. Hence, this study was aimed to determine if plasmid-less isolates of CT is present in India. Endocervical swab samples collected from 311 symptomatic women were screened for CT using real-time PCR targeting ompA gene and plasmid (pCT), culture and direct fluorescent assay (DFA). Of 311 samples, 57 (18.32%) were positive for both pCT and ompA and 19 samples (6.1%) were positive for pCT only. Samples detected positive for both pCT and ompA real-time PCR was significantly higher ($p<0.001$) than using either of the single test. One sample was detected positive for ompA but not for pCT and was subsequently confirmed by DNA sequencing, southern blotting and culture as plasmid-less isolate. The existence of plasmid-less isolate of CT in India demands the use of real-time PCR assays for better clinical management.

INTRODUCTION

Genital tract infections due to Chlamydia trachomatis (CT) are a major cause of morbidity in sexually active individuals worldwide and in developing countries including India. A high prevalence of female genital chlamydial infection has been previously reported from India (Singh et al., 2002; Joyee et al., 2004). In women untreated infections may progress to serious reproductive sequalae including ectopic pregnancy, pelvic inflammatory disease and salpingitis with tubal scarring and infertility (Low et al., 2006). Therefore, early and accurate diagnosis is necessary to prevent these complications and thereby controlling the spread of infection.

Several studies have shown that nucleic acid amplification tests are far superior to conventional tests for diagnosis of CT infection. Polymerase chain reaction (PCR) based tests that detect CT specific DNA in endocervical swab samples have been developed and evaluated (Chan et al., 2000; Jensen et al., 2003). Real-time PCR for detection of Chlamydiae is considered highly sensitive and has a high degree of specificity over conventional PCRs (Hardick et al., 2004; Boel et al., 2005).

The evolutionary conserved, 7.5- kb CT cryptical plasmid (pCT) is present in seven to ten copies per cell and is the target of most molecular diagnostic tests. Inspite of being under positive selection pressure, several clinical isolates of Chlamydia sp. have been documented worldwide, which lack the cryptical plasmid (Peterson et al., 1990; An et al., 1994; Farencena et al., 1997). These isolates are crucial for research to gain an understanding of the functions of the plasmid and its contributions to the morphology, development and pathogenecity of this bacterium. Hence this study was aimed at screening for plasmid-less isolates of CT in India using dual target (plasmid and ompA) real-time PCR assays.

MATERIALS AND METHODS

STUDY POPULATION AND SAMPLING CONDITIONS

311 symptomatic women (age range 20 to 40 years) attending Gynecology outpatient department of Safdarjung Hospital, New Delhi, India were enrolled for the study. Prior written consent was obtained from each patient and the study was approved by the hospital Ethical Committee. The cervix was inspected for ulcers, warts, ectopy, erythema, discharge, and lesion if any. After cleaning the exocervix with cotton swab (Hi Media, India), endocervical swabs were collected in sterile vials containing phosphate buffered saline for

diagnosis of CT infection by real-time PCRs and cell culture .

REAL-TIME PCR CYCLING CONDITIONS

Chlamydial DNA was extracted from all clinical samples and confirmed for positivity by a human beta () - globin PCR as described previously (Singh et al., 2003). All -globin PCR positive samples were diagnosed for CT by RealArt Standard kit and modified RealArt Plus kit (modified by removal of ompA specific primers and probes) (QIAGEN Diagnostics GmbH, Germany) targeting 106 bp of ompA gene and 111 bp fragment of pCT. To determine the analytical sensitivity of the RealArt Plus kit, plasmid dilution series was set up from 0.66 to 0.002 copy equivalents µl $^{-1}$ and for the ompA based RealArt Standard kit from 10 to 0.078 copy equivalents µl $^{-1}$ according to standard protocols. All real time PCRs were run on the Rotor-Gene™ 3000 instrument (Corbett Research, Australia).

CULTURE OF CT CLINICAL ISOLATES

Real-time PCR positive samples were cultured in McCoy cell line (National Centre for Cell Sciences, Pune, India), harvested at 66 hours post infection and subsequently examined for positivity by culture confirmation test DFA as described previously (Mittal et al., 1993). Cultures were harvested and chlamydial pellet was dissolved in appropriate quantity of Sucrose Phosphate Glutamate Buffer (4X) and stored at –180 ° C for further use.

CONFIRMATION OF PLASMID-LESS CLINICAL ISOLATE OF CT

Along with culture confirmation, the specificity of ompA real-time PCR amplicon of the plasmid-less clinical isolate was determined by DNA sequencing using BigDye Terminators cycle sequencing kit (Applied Biosystems, USA) according to manufacturer's instructions on 3130xl Genetic Analyzer (Applied Biosystems, USA). Further non-radioactive Southern blotting analysis was performed on plasmid-less clinical isolate using digoxigenin labeled probes against 517 bp plasmid amplicon as described previously (Roymans et al., 1996). Appropriate positive and negative controls were included in the experiments. Positive control comprised of pCT positive sample cultured in McCoy cells whereas negative control consisted of uninfected McCoy cells.

STATISTICAL ANALYSIS

PriProbit ™ version 1.63 (Kyoto University, Japan) was used to determine the analytical detection limit of real time PCRs. The statistical significance for performance of real-time assays was calculated using McNemar test.

RESULTS

All 311 samples were found to be positive for -globin PCR. Out of 311 samples, 57 (18.32%) samples were positive for both pCT and ompA real-time PCRs and 19 (6.1%) samples were positive by pCT real-time PCR only (Table 1). Moreover, the number of samples detected positive for both pCT and ompA real-time PCR were significantly higher ($p<0.001$) than detected using either of the single test. The analytical detection limit was 0.2 copies µl $^{-1}$ ($p<0.05$) and 0.5 copies µl $^{-1}$ ($p<0.05$) for pCT and ompA real-time PCRs respectively. All the 77 samples detected positive for real-time PCR were also found positive in culture system.

Figure 1

Table 1: Detection of *C. trachomatis* in endocervical swabs by real-time PCRs using plasmid and *omp A* as target.

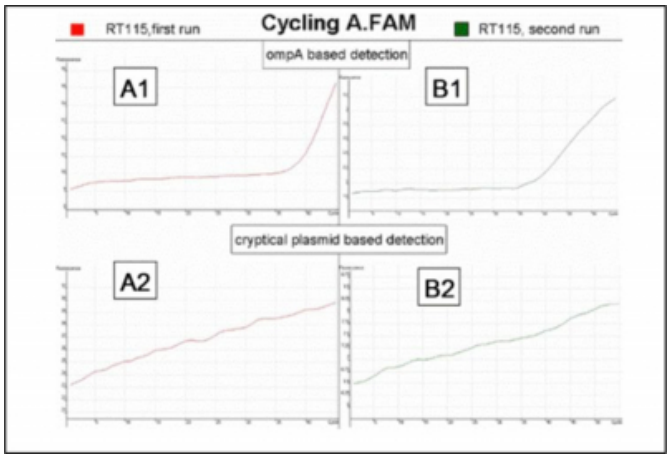
Real-time PCRs	N=311 (%)
pCT +ve/ *omp A* +ve	57(18.32)*
pCT +ve/ *omp A* -ve	19(6.10)
pCT -ve/ *omp A* +ve	01(0.32)
pCT -ve/ *omp A* -ve	234(75.24)

* By using McNemar test, positivity for CT was significantly higher ($p<0.0001$) using plasmid and *omp A* as targets.
( ) Figures in parentheses denotes percentages.

OmpA CT real-time PCR (Fig 1a. Panel A1, B1) was able to detect a probable plasmid- less isolate of urogenital CT as pCT was not detected in the same run by using the pCT real-time PCR (Fig 1a. Panel A2, B2).

Figure 2

Figure 1a: Identification of plasmid-less isolate of



The results of two real-time PCR runs (panel A and B) of sample RT115 on the Rotor-Gene 3000 instrument are shown. Depicted in the figure is the C. trachomatis specific detection channel (FAM). Sample RT115 was analyzed by using an ompA based assay (panel A1 = first run, panel B1 = rerun of this sample) and an cryptical plasmid based assay (panel A2 = first run, panel B2 = rerun). In both PCR runs inhibitions of this sample were not observed (data not shown). Patient sample RT115 was repeatedly shown to be C. trachomatis positive by using the RealArt Standard kit (ompA based, panel A1 and B1) and negative by using the modified RealArt Plus kit (cryptical plasmid based, modified by removal of ompA specific primers and probes, panel A2 and B2).

DNA sequencing of ompA real-time PCR amplicon (106bp) of the plasmid-less clinical isolate confirmed its specificity (Table 2).

Figure 3

Table 2: BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) and Taxonomy Report of DNA sequencing of amplified plasmid-less isolate DNA using real time PCR.

Taxonomy Report

BLASTN 2.2.12

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

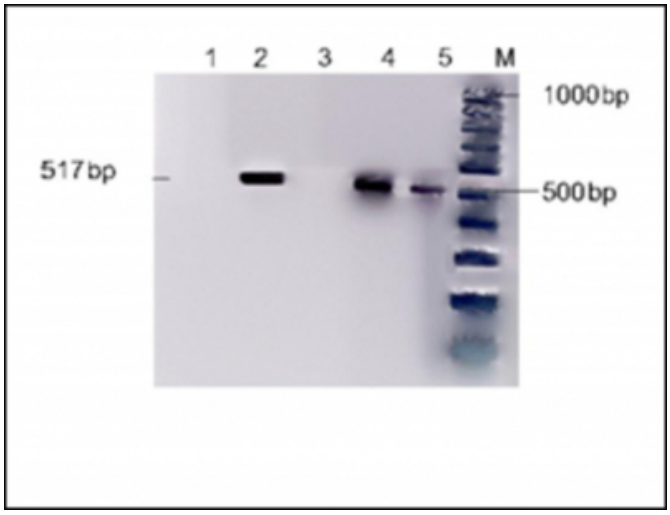
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences)
3,540,011 sequences; 15,726,889,226 total letters

root	100 hits	7 orgs	
. Chlamydia	99 hits	6 orgs	
[cellular organisms; Bacteria; Chlamydiae/Verrucomicrobia group; Chlamydiae; Chlamydiae (class); Chlamydiales; Chlamydiaceae]			
. . Chlamydia trachomatis	77 hits	3 orgs	
. . . Chlamydia trachomatis A/HAR-13 ..	1 hits	1 orgs	
. . . Chlamydia trachomatis D/UW-3/CX .	1 hits	1 orgs	
. . Chlamydia suis	17 hits	1 orgs	
. . Chlamydia muridarum	5 hits	2 orgs	
. . . Chlamydia muridarum Nigg	1 hits	1 orgs	
. synthetic construct	1 hits	1 orgs	[other sequences; artificial sequences]

The specificity of plasmid-less clinical isolate was further confirmed by non-radioactive southern blotting analysis using digoxigenin labeled probes against 517 bp plasmid PCR amplicons. The results of southern blotting analysis are shown in fig. 1b.

Figure 4

Figure 1b: Southern blotting hybridization for confirmation of plasmid-less isolate of using 517 bp pCT PCR product and digoxigenin labeled probes



Lane 1- Negative control (uninfected McCoy cells). Lane 2- Positive Control (pCT positive sample cultured in McCoy cells). Lane 3 - Sample negative for pCT for modified RealArt Plus kit assay and positive for ompA using the RealArt standard kit. Lanes 4 and 5- Samples positive for modified RealArt Plus kit. Lane M- 100 bp DNA ladder (Bangalore Genei, Bangalore, India).

DISCUSSION

In the present study a highly specific and sensitive real-time PCR was used to detect the presence of plasmid-less urogenital isolate of CT in symptomatic female patients. Out of 311 samples screened in the study, 57 (18.32%) were positive for both pCT and ompA real-time PCR and 19 samples (6.1%) were positive for pCT only. One sample was detected positive for ompA but not for pCT and was subsequently confirmed by DNA sequencing, southern blotting and cell culture as plasmid-less isolate. Due to lack of a national screening program there was no such accountability till date about the prevalence rates of such isolates of CT in the semi-urban and rural population of India. The patient harbouring this isolate was a resident of New Delhi, the capital of India and a major cosmopolitan city in India. Since tourism, business and educational exchanges between India and the world have seen a rapid increase in the last decade there is a huge exchange of global populations from different geographical regions. Hence the identification of plasmid-less CT strains in the Indian sub-continent should be pondered upon with caution and efforts should be made to stop the cross pooling of such isolates.

Reports of a genetic variant of CT in Europe are thrilling and have provided several lessons (Herrmann, 2007; Schachter, 2007). Ripa et al (Ripa and Nilsson, 2006) reported deletion of 377 base pairs in the pCT target area using Abbott and Roche commercial kits, which detected false CT negatives and found a variant that was positive using RealArt Standard kit. The high copy number (7-10 copy cell $^{-1}$) pCT is universal among all strains of CT and hence serve as targets for nucleic acid amplification tests along with 16S rRNA and ompA gene. However, the identification of plasmid-less isolates of CT stress upon the fact that there is need for designing nucleic acid tests which simultaneously target the pCT for its multiple copy number and the genome for negating the probability of false negative results while detecting CT. Use of commercial assays which exclusively detect CT plasmid sequences, provides a further reason for caution - ongoing or new therapies based on these diagnostic kits will ONLY eliminate plasmid bearing strains, whereas plasmid-less strains will remain undetected and may accumulate in the population.

Presence of plasmid-less isolates in nature (Peterson et al., 1990) can help understand Chlamydia biology in several ways .The fact that pCT is positively selected during evolution confers the fact that it provides advantages to the pathogen for host infection, replication and survival by modulation of host's immune system. Hence, availability of such strains would favour further studies in pCT's role in pathogenesis of the organism.

In conclusion, this study suggests that the national impact of existence of a plasmid-less isolate of CT should be looked at for better clinical management of this infection. The use of this real-time PCR kit will help in reviewing the prevalence rates of endocervical CT in the Indian population and the rest of the developing world. The spread of new variant of CT differs between countries but the reason for this remains unexplained and should be explored in the future. Further data is still needed to determine the size of the problem of emerging CT variant in India since only symptomatic patients were screened in our study.

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